XJENZA ONLINE

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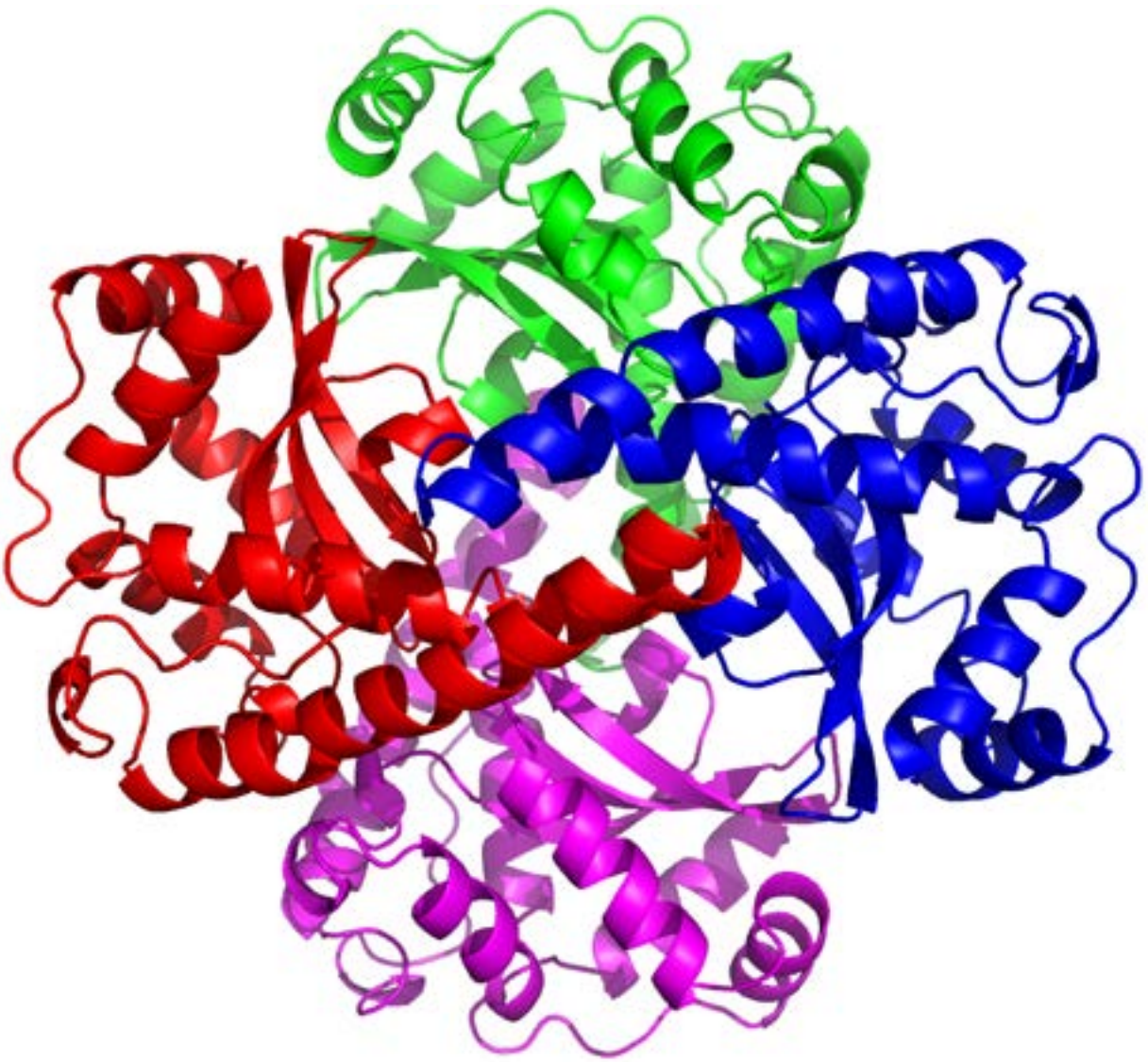

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The Journal of the Malta Chamber of Scientists

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.

Notes are fully referenced, peer-reviewed short articles limited to three journal pages that descrivenew theories, concepts and developments made by the authors in any branch of science and technology. Notes need not contain results from experimental or simulation work.

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.

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Manuscripts should be sent in electronic format (via e-mail) to the Editor of Xjenza:

Prof. Giuseppe Di Giovanni
Department of Physiology and Biochemistry
Faculty of Medicine and Surgery (On Campus)
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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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The work described in the submitted manuscript must have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (http://www.wma.net/en/30publications/10policies/b3/index.html); EU Directive 2010/63/EU for animal experiments (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm); Uniform Requirements for manuscripts submitted to Biomedical journals (http://www.icmje.org). This must be stated at an appropriate point in the article.

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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Preparation of Manuscripts
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.
- Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, including post-publication. Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and complete postal address. Contact details must be kept up to date by the corresponding author.
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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.

Footnotes
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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Indicate each footnote in a table with a superscript lowercase letter.

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- Make sure you use uniform lettering and sizing of your original artwork.
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- Only use the following fonts in your illustrations: Arial, Courier, Times, Symbol.
- Number the illustrations according to their sequence in the text.
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- Provide captions to illustrations separately.
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Formats
Regardless of the application used, when your electronic artwork is finalised, please 'save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):
EPS: Vector drawings. Embed the font or save the text as 'graphics'.
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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Citation in text

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.

Web references
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.

References in a Special Issue
Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

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;
2. Two authors: both names and the year of publication;
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.

Journal Abbreviations Journal names should be abbreviated according to:
-Index Medicus journal abbreviations: http://www.nlm.nih.gov/tsd/serials/lji.html;
-List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php;
-CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

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Xjenza accepts video material and animation sequences to

support and enhance the presentation of the scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labelled so that they directly relate to the video files content. This should be provided in one of our recommended file formats with a preferred maximum size of 50 MB.

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

Use of the Digital Object Identifier

The Digital Object Identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document, particularly 'Articles in press' because they have not yet received their full bibliographic information. When you use a DOI to create links to documents on the web, the DOIs are guaranteed never to change.

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Formerly *Xjenza*

Xjenza Online - Journal of Malta Chamber of Scientists
www.xjenza.org

Editorial

Giuseppe Di Giovanni
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Dear Readers,

Welcome to the new issue of *Xjenza Online*, in which we have gone from strength to strength!

This year marks the third issue of Xjenza Online, the official journal of the Malta Chamber of Scientists and its Physiological Society (MPS). The first year has been a year of transition. No longer in its infancy, Xjenza Online has taken on an important role in the Maltese scientific community. The journal has generated enormous interest amongst residents, faculty and alumni in contributing to the Journal. We have created a rigorous peer-review process of which the Journal can only be proud of. From the outset, the goal of this first year was to maintain our position, yet expand the scope, visibility and credibility in the scientific community. The first aim of this mission was commitment to a centralized, all-electronic submission, international review and publishing platform. This has not been achieved without growing pains, but has brought us one step closer to the standards set by other peer-reviewed publications. We are already hard at work on building a better, leaner platform for the years to come. Our second goal, the most ambitious one, was to achieve bibliographic database listing for our scientific articles. Scopus, Google Scholar, Medline and Web of Science are the most used by researchers. This is the *sine qua non* of legitimacy for a scientific journal. Thanks to the hard work of the editorial team, we are now ready to submit our application to the Scopus committee. The next steps will then be PubMed and Web of Science. The bibliographic database listing will greatly improve our appeal for authors and will undoubtedly increase the quality and quantity of submissions in the future.

Finally, we want to expand the reach of the Journal. Two print copies of the previous issue have been donated to the Director Library Services, Mr. Kevin J. Ellul of our library. Now Xjenza Online is listed in the UoM catalogue and a print copy available at the Melitensia Department and at the Health Sciences Library. Furthermore, Xjenza's previous website that was linked to the Library's on-line catalogue has been replaced by the new Xjenza website. The journal can be searched through HyDi by typing Xjenza and narrowing down the "All Items" option to journals and "anywhere in the record" in the title. After summer, the national repository will be active for all the periodicals printed in Malta and Xjenza Online will be included. A copy of the previous issue has been sent to Malta Council of Science and Technology (MCST) Chairman Dr Jeffrey Pullicino Orlando and to all the Head of Departments of the University of Malta. Notably, this issue was sent to COST's 35 European Member Countries' members that participated in the COST meeting held in Malta last October, who also sponsored the issue printing. Beyond highlighting the accomplishments of the local researchers, this will serve as a springboard for the journal to receive contributions from a wider base and from different countries in the future, putting us on a par with other highly recognized institutional journals.

This issue of Xjenza Online again offers a wide range of high-quality articles from national and international researchers. Dr Portelli, a Maltese researcher who works in Belgium at Vrije and Ghent University, emphasizes the important new role of Ghrelin in epilepsy and in drug discovery in her review. This article is an updated summary of her talk given at the 3rd Neuroscience Day @ the University of Malta at the end of November 2012.

The second contribution is from Professor Holger Mitterer, a German researcher who moved recently

to Malta University, on psycholinguistics and phonetics. Mitterer showed how phonetic alignment in conversation is a more complex process than most current theories seem to suggest. Strikingly, failure to align may not impede mutual understanding.

The next article by Dr Fenech and Dr Grech addresses the important subject of pharmacogenetic-guided, patient targeted therapy that has now become the developing fulcrum of personalized medicine, as it provides the best means to optimize benefit/risk ratio in pharmacological management.

Optimism bias is a well-established psychological phenomenon that has implications from mental health to economic theory. Dr Bajada reviews this interesting field of new research starting from cognitive neuroscience and neuroimaging data on the neural basis of the optimism bias. He provides a psychological and neuroscientific grounding for the optimism bias in the treatment of depression.

Dr Gilananzé's research note is on the use of silk and acellular dermal matrices grafts as viable alternatives to autologous skin transplantation. The silk matrix seemed to be a good candidate for the development of skin structures, although studies have outlined the fact that raw silkworm silk can be immunogenic.

I would like to draw your attention to the next article by Professor Gary Hunter and colleagues that focuses on X-ray crystallography. Hunter and co-workers highlight the methodologies used in protein expression, purification and the determination of the three-dimensional protein structures by X-ray crystallography. The front cover image of this issue is the three-dimensional structure of SOD-2, one of two MnSODs proteins from Caenorhabditis elegans isolated in Therese and Gary Hunter's laboratory in Malta.

Jonathan Henwood describes in his article the less common variant of Chara vulgaris the C. vulgaris var. papillata that has been recorded for the first time locally in mats of Chara vulgaris examined from il-Qattara pool and Qawra (Dwejra, Gozo).

From the researchers who participated in the second annual Science in the House at the Grand Master's Palace in Valletta on 26 September 2013, Nigel Borg and co-authors contributed to this issue with a research article on Akt, a Serine/Threonine protein kinase, which mediates growth factor-associated cell survival. Constitutive activation of Akt (phosphorylated Akt, P-Akt) has been observed in several human cancers, including breast cancer and may be associated with poor prognosis and chemotherapy as well as radiotherapy resistance. Borg et al. showed that a subset of Triple Negative Breast Cancer Patients in Malta, consisting of 26% of cases, have a moderate to high activation of Akt. This subset would be eligible for therapies targeting the PI3K/Akt pathway. This would be of great importance due to a current lack of effective therapies against triple negative breast cancer cases. Alexandra Fiott and colleagues explore the possibility that absolute HbA1C improves the genotype-phenotype association in Type 2 Diabetes. In vitro glycation showed that Hb BetaValletta, found in 1.8% of Maltese adults, does not infuence glycation and thus the HbA is not influenced by this variant in heterozygotes/homozygotes. Finally, Grech and Borg give an exhaustive description of the control of globin gene expression by Kruppel-like factors, a family of DNAbinding transcriptional regulators that are involved in a wide range of biological processes.

Figure 1: The E-i-C of *Xjenza Online* Prof. Giuseppe Di Giovanni donating a copy of the Journal (Vol.1 issue 2) to the Director Library Services, Mr. Kevin J. Ellul. On the left Mrs Joanna Felice, Deputy Director Library Services on the right Dr Duca Edward, secretary of the Malta Chamber of Scientists

The next article is on seismology. Agius and colleagues investigated the data availability and quality of the currently only seismic station on Malta since its installation in 1995, and established spectral patterns in the seismic data. The results are important for the future deployment of permanent seismic stations on the Maltese islands, and for the analysis of local seismic hazard and ground motion studies

The final standard article in the current issue is a review of the factors influencing the male to female ratio at birth. Males usually predominate and while

stress decreases the ratio, wellbeing and good health tends to increase it. This paper reviews the multitudes of factors that have been implicated as acting this ratio, from historical times to date.

Lastly, three pieces of News by David Magri on Science in the House 2013, 1st Lecturer's Mini-Symposium held on January 30th, 2014 and COST Action Chemistry Conference on Supramolecular Chemistry in Water highlight important happenings in our scientific community.

I would like to finish by thanking the authors and reviewers.

None of this would have been possible without the support of our editors and members of the journal board.

We hope that this second volume issue reminds us all of what makes the Malta Chamber of Scientists so special: great science, great friendship and commitment to excellence that never wavers.

Giuseppe Di Giovanni
Editor-in-Chief

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Doi: http://dx.medra.org/10.7423/XJENZA.2014.1.01

Review Article

Neuropeptide receptors as potential antiepileptic drug targets: focus on the ghrelin axis

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[2]Laboratory for Clinical and Experimental Neurophysiology, Neurobiology and Neuropsychology (LCEN3), Department of Neurology, Institute for Neuroscience, Ghent University (UGent), Belgium

Epilepsy is a very serious neurological disorder which is often underrepresented. Around 50 million individuals worldwide have active epilepsy with recurrent seizures and in spite of the medical advances over the years, 30% of these patients remain as drug resistant (Pati and Alexopoulos, 2010). Even after several years of research, there is still a lack of good understanding on the pathophysiology of seizure disorders (Perucca, 2011). Investigators in this field believe that there is a great need for novel antiepileptic drugs (AEDs) that act differently than the drugs available on the market. The majority of AEDs act by blocking sodium channels (phenytoin, carbamazepine) or by the augment of GABAergic transmission (phenobarbital, valproic acid). A newer generation of AEDs has expanded therapeutic options, however these are not superior to the older drugs (Hitiris and Brodie, 2006). Patients with mesial temporal lobe epilepsy (mTLE) are among the most pharmacoresistant to these medications (Pati and Alexopoulos, 2010). In order to attempt the rectification of this dilemma, the neuropharmacologist needs to not only try and find AEDs with new mechanisms of action, but to also keep in mind what information is currently available on the pathophysiology of epilepsy. It is clear that during the complicated process of epileptogenesis, several different mechanisms are taking place, thus one should ideally identify new compounds that are capable of targeting different pathways simultaneously. The focus of epilepsy researchers is to identify compounds that are not only capable of attenuating seizures (anticonvulsant), but are also antiepileptogenic (can prevent epilepsy) or disease-modifying (halting its progression).

A lot of interest is being shown towards neuropeptides as a way to suppress epileptic seizures. A number of neuropeptides have been extensively studied in the pathogenesis of epilepsy, such as neuropeptide Y, galanin and somatostatin. However the poor ability of these neuropeptides to penetrate the blood brain barrier (BBB) serves as the main obstacle for brain drug development (Robertson et al., 2011). Neuropeptide-based treatments present a number of advantages in comparison to treatments that target classical neurotransmitter systems and ion channels, both in efficacy as well as safety (Hokfelt et al., 2003; Portelli et al., 2012a). Since neuropeptides are normally released from neurons in the presence of high frequency firing or pathological conditions, a likely advantage is that the clinical effects of neuropeptide receptor antagonists will only become evident under epileptic conditions where high frequency firing is involved, which in turn reduces the risk of adverse effects. Ghrelin, a pleiotropic peptide that has excited the scientific community generally since its discovery in 1999, has recently been introduced in the field of epilepsy.

Ghrelin is produced both centrally and peripherally. Ghrelin requires modification on the serine-3 by O-acylation with octanoate in order to bind to the G-protein coupled receptor (GPCR) growth hormone secretagogue receptor type 1a (GHSR1a) (Kojima et al., 1999). The ghrelin receptor gene encodes two types of GHSR mRNA, known as 1a and 1b, resulting in

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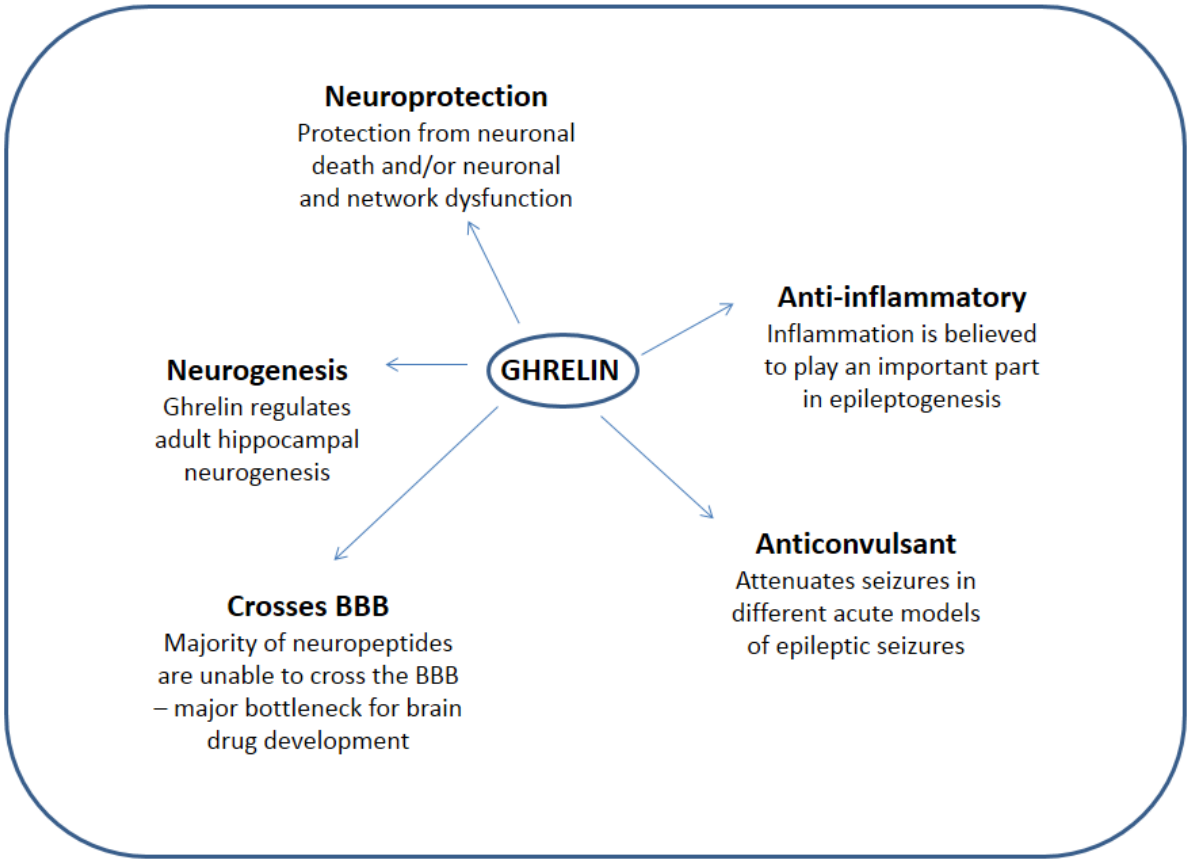

Figure 1: Some of the known effects of ghrelin, which makes this neuropeptide system a promising target for antiepileptic and antiepileptogenic treatments.

two isoforms: GHSR1a and GHSR1b (Camina, 2006). GHSR1a is given more importance, since GHSR1b is unable to bind or be activated by ghrelin. GHSR1a, as with ghrelin, is widely expressed both centrally and peripherally, including in seizure-prone regions such as the hippocampus. For a more detailed account of the cell biology of the ghrelin receptor, please refer to the review by Camina (2006).

In the past six years the ghrelin system has been reported to have anticonvulsant properties. Ghrelin has been shown to have an inhibitory effect on seizures induced by pentylenetetrazole (Obay et al., 2007; Obay et al., 2008), penicillin (Aslan et al., 2009), and kainic acid (Lee et al., 2010), but its anticonvulsant mechanism of action has remained elusive. Recently we have attempted to unravel ghrelin's anticonvulsant mechanism of action using the in vivo rat model for pilocarpine-induced limbic seizures, the mouse pilocarpine tail infusion model, transgenic mice with a GHSR deletion, electrophysiology in hippocampal slices, EEG recording in freely moving rats, and HEK293 cells expressing the human GHSR, to determine inverse agonism, activation, desensitization, internalization and resensitization (Portelli et al., 2012b). Ghrelin and the ghrelin-mimetic capromorelin attenuated pilocarpine-induced seizures in rats and mice. Experiments with transgenic mice established that ghrelin requires the GHSR for its anticonvulsant effect. Interestingly we found that $GHSR^{-/-}$ mice had a higher seizure threshold than $GHSR^{+/+}$ mice when administered the muscarinic agonist pilocarpine. This prompted us to look further into pharmacological modulation of the receptor, where we discovered that abolishing the constitutive activity of GHSR by inverse agonism results in the attenuation of seizures and epileptiform activity. We verified that ghrelin's potential to rapidly desensitize the GHSR is followed by internalization of the receptor and a slower resensitization process. This, together with our present novel findings that different ghrelin fragments possess similar agonistic potencies but different desensitization characteristics on the GHSR, led us to elucidate that ghrelin probably attenuated limbic seizures in rodents and epileptiform activity in hippocampal slices due to the desensitizing effect on the GHSR (Portelli et al., 2012b). This in turn constitutes a novel mechanism of anticonvulsant action whereby an endogenous agonist reduces the activity of a constitutively active receptor.

Ghrelin presents a number of advantages when compared to other well-established anticonvulsant neuropeptides. This neuropeptide can easily cross the BBB, and the ghrelin system has been attributed to affect a number of physiological processes, ranging from its potent anti-inflammatory and neuroprotective properties to its ability to protect the BBB and induce hippocampal neurogenesis (Moon et al., 2009; Portelli et al., 2012a) (Fig 1). Studies have shown that during the process of epileptogenesis, all previously mentioned physiological processes are negatively affected (Ravizza et al., 2006; Boer et al., 2008; Choi and Koh, 2008; Ravizza et al., 2008; Vezzani et al., 2011; Zlokovic, 2008; Coremans et al., 2010; Marchi et al., 2012; Parent and Kron, 2012; Vezzani et al., 2013). Recently, a clinical phase III trial for the ghrelin receptor agonist JMV1843 (Macimorelin), indicated for growth hormone deficiency in humans, has been completed and the drug was found to be well tolerated. It is now currently being developed by the company Æterna-Zentaris.

Our knowledge on the role of the ghrelin axis in the pathogenesis of epilepsy is still in its infancy. Targeting the ghrelin receptor has been shown to attenuate acute seizures in different models (Obay et al., 2007; Obay et al., 2008; Xu et al., 2009; Lee et al., 2010; Portelli et al., 2012a; Portelli et al., 2012b), and from what is already known with regard to this system's properties in view of inflammatory cascades, neuroprotection, neurogenesis, and BBB protection, it is promising that the ghrelin axis could play a role in the process of epileptogenesis.

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Research Article

Agreeing to disagree: Constant non-alignment of speech gestures in dialogue

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Abstract. Numerous studies suggest that interlocutors in a dialogue align with each other in terms of their articulatory gestures. It is often suggested that this, first, is the consequence of an automatic tendency for imitation and, second, this fosters mutual understanding. Making use of online archives of media, it was tested whether alignment is hence inevitable. The focus was on the pronunciation of the German word *ist* (Engl., 'is'). The standard pronunciation is [ɪst], but speakers with a Swabian accent produce [ɪʃt], acoustically reflected in the fricative spectra. We measured the spectra of fricatives in *ist* from interviewers while interviewing either a prominent German politician using the Swabian variant or an interviewee using the standard variant. Results showed neither an overall influence of the interviewees' pronunciation on the fricative realization by the interviewer nor a tendency to align over time for interviewer-interviewee pairs with different pronunciations. This shows that phonetic alignment in conversation is a more complex process than most current theories seem to suggest. Moreover, failure to align may not impede mutual understanding.

1 Introduction

While speech production and speech perception are typically studied as separate phenomena, the speech we hear influences how we speak. A seminal study by (Harrington et al., 2000) showed that speech production remains flexible over the lifespan and adapts to the ambient speech see also (Sancier and Fowler, 1997). Numerous studies have shown similar effects in laboratory settings, with imitation occurring over the time span of a conversation (Pardo, 2006) and in laboratory tasks such as shadowing (Fowler et al., 2003; Mitterer and Ernestus, 2008; Shockley et al., 2004). In a broader framework, (Pickering and Garrod, 2004) suggested that such alignment effects occur on many levels in interactive dialogue and that they underlie the ease with which interlocutors achieve parity, see also (Miller et al., 2010).

Phonetic alignment has been found with both phonetic measurements and native listeners' judgments. (Fowler et al., 2003), for instance, measured the voice onset time (VOT) of stop-vowel syllables recorded during a shadowing task. The to-be-shadowed stimuli had a long or a short VOT, and this affected the VOT in the shadowing responses. (Brouwer et al., 2010) measured the duration and the degree of segment reduction for shadowed utterances in response to more or less casual speech. They found that shadowing responses to strongly casual speech were shorter and contained more segment reductions than responses to more formal speech. Another frequently employed method to test for phonetic alignment is a perceptual matching task (Goldinger, 1998). In this task, participants hear utterances from a given speaker recorded in a baseline condition or recorded during or after conversation with a reference speaker. Participants typically judge the utterances recorded during or after the conversation as more similar to utterances from the reference speaker than the baseline recordings (Pardo, 2006), indicating phonetic alignment.

Maybe due to the practice of "null-hypothesis significance testing", in which the absence of alignment is nothing more than an uninformative null-effect, the emerging picture seems to be that one adapts to all aspects of an interlocutor's speech. As (Miller et al., 2010) (p. 1615) state "[i]n summary, speech alignment occurs

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on both phonetic and extraphonetic levels". Intuitively, however, it seems unlikely that one would align with all aspects with an interlocutor, independent of the difference between the interlocutor's and one's own pattern. An obvious example here is a foreign accent. It seems unlikely that a native speaker would incorporate aspects of a foreign accent in one's own speech.

Indeed, recent studies with both acoustic and perceptual measures of alignment often report perceptual alignment but no acoustic alignment (Pardo et al., 2013). One possible reason for this may be that there were no active manipulation of phonetic differences between stimuli and responses, that is, speakers and shadowers were from the same sociolinguistic background and there was no a-priori defined phonetic difference between the two groups. It may be necessary to start with such differences. Indeed, another study (Nguyen et al., 2012) investigated a dialect difference between different versions of French and found evidence for convergence. Importantly, this study showed that Northern French speakers, considered to be the standard, adapt to non-standard Southern French speakers. This raises the question whether convergence in terms of speech gestures used for a given segment is inevitable. We identified a source of naturalistic data to test this in German fricative productions. German contrasts an alveolar fricative [s] with a post-alveolar fricative [ʃ] (similar to the English minimal pair 'sea'-'she'). In fricative-stop clusters, however, the contrast is neutralized, and Standard German uses only [ʃt] in onset position (e.g., 'Stein' / ʃteɪn/, Engl. 'stone') and only /st/ in coda position (e.g., 'Faust' /faust/). Swabian speakers from the Southwest of Germany, however, use [ʃt] in both cases. The difference between the two fricatives is captured well by a measurement of the spectral center of gravity. For /s/, the tongue is close to the teeth, and the resulting small space gives rise to high-frequencies, while for /ʃ/, the tongue is further back, leaving more space between tongue tip and teeth, given rise to lower frequencies: Just as for musical instruments, more space means lower frequencies. The center of gravity is something like the "average" frequency of the signal and is hence lower for /ʃ/ than for /s/. Given this acoustic correlate, it is straightforward to measure whether there is alignment or not.

If there is alignment when a Standard German speaker interacts a Swabian speaker, either the standard speaker, who regular produces [st] coda clusters, should produce fricatives in these clusters with an increasingly lower spectral center of gravity—becoming more [ʃ] like, or the Swabian speaker, who regular produces [ʃt] coda clusters, should produce fricatives with an increasingly higher center of gravity—becoming more [st] like.

One way to test for phonetic alignment is by exploiting online media archives (e.g., (Gregory and Webster, 1996)). Interestingly, a prominent German politician, Wolfgang Schäuble, PhD, tends to use the Swabian pronunciation of the coda cluster *-st*, while interviewers typically use the standard variant. Making use of large online media corpora, we compared interviews in which a given interviewer interacted with Dr. Schäuble with interviews in which the same interviewer interacted with a speaker using the standard German variant. By comparing the same interviewers over different interviews, we can see whether the interviewee's divergent phonetic choices influence the phonetic choices of the interviewer. Ideally, one would also compare the behavior of the interviewee when confronted with Standard German and Swabian German interviewers. This is, for obvious reasons, not possible, because there are only very few interviewers that use a regional accent. Moreover, it is more likely that the interviewers will align with Dr. Schäuble than vice versa. (Gregory and Webster, 1996) found that social status influences the direction of alignment, so that the interviewer will align with high-status guests while lower-status guests align with the interviewer. Dr. Schäuble is regarded as one of the intellectual heavy weights in German politics and is respected across the political spectrum[1]. It should hence be more likely that the interviewers should align than vice versa. As a test of whether alignment is automatic, we focus on just the way the fricative in a high-frequency word is produced. The logic is that, if alignment is automatic and there is a clear difference in speech gestures (alveolar versus post-alveolar), alignment should occur. It is important to note that a failure to find alignment here does not preclude that other aspects, especially in the domain of prosody, may align. However, in the discussion of the perception-production link, speech gestures have figured prominently (Fowler, 1996; Ohala, 1996), so that a failure to find alignment in this respect is theoretically meaningful.

2 Methods

An online search resulted in seven interviews of more than five minutes with Dr. Schäuble (see Table 1). For each of these interviews, a control interview was identified in which the same interviewer conversed with an interviewee that used the standard variant. All interviewers and control interviewees spoke standard German with no local coloring. Given that the acoustic properties of fricatives are strongly influenced by surround-

[1]To provide one example of this, it is worthwhile to consider the debate about the capital of Germany after the reunification, the so-called Hauptstadtdebatte. In a parliamentary sitting in which there was no voting among party lines, it is widely assumed that Dr. Schäuble's speech tipped the scale in favor of Berlin, even though it had been anticipated that Bonn, the capital of former West Germany would stay the capital after reunification.

ing vowels (e.g., (Smits, 2001)), we focused on productions of the (very) high-frequency word German word *ist* (Engl. 'is'). In each interview, all occurrences of *ist* were annotated if they had a clear fricative portion. Tokens were rejected if the fricative was phonetically voiced or when the fricative was followed by another fricative with no clear boundary[2]. Additionally, up to ten instances of the phonetic string [ɪʃ] and [ɪs] were identified in words that were not associated with any geographical or sociolinguistic differences[3]. These tokens served to indicate whether the fricative in ist is more similar to a speaker's alveolar /s/ or post-alveolar /ʃ/. This comparison is important, because fricative spectra can vary strongly between speakers due to factors such as articulator size and also between recordings because of different levels of ambient and system noise during the recordings as well as the high-frequency cut-off of a recording.

3 Results

First, it was checked whether the control interviewees indeed showed the Standard German pattern with a fricative in *ist* that is similar to their alveolar [s]. This was clearly the case. The control interviewees produced a fricative in *ist* (mean CoG: 5887 Hz) that is similar to their /s/ (mean CoG: 5838 Hz) but differs from their /ʃ/ (mean CoG: 4010 Hz). A linear mixed-effect model with the /s/ mapped on the intercept showed that the fricative in *ist* was not different the other /s/'s ($b_{\text{Fricative="}ist\text{"}} = 46$, t = 0.56), but that the difference between /s/ and /ʃ/ was significant ($b_{\text{Fricative=/ʃ/}} = -1823$, t = 5.57). Having established this, it can now be examined whether the fricative productions from the Swabian speaker Dr. Schäuble indeed deviate from the standard pattern, and whether this in turn influences the interviewers. Table 1 shows the individual means and Figure 1 the overall means for the fricatives' center of gravity (CoG) for tokens of *ist*, tokens of [ɪʃ], and tokens of [ɪs] for the Swabian speaker in different interviews and the respective interviewers' CoGs in conversation with this Swabian speaker or a speaker with the standard pattern. The means show that the interviewers produce a fricative in *ist* that is similar to their [s] in both types of interviews, while the Swabian speakers shows the expected deviant pattern, with a fricative in *ist* that is more similar to the [ʃ].

Table 1: Individual mean spectral centre of gravity for the fricatives by interviewers conversing with a Standard speaker or a Swabian speaker. Note that level difference between different interviews by the same speaker are caused by recording quality.

Interview by (duration in s)	**interviewer/ist/-exposed**			**interviewer/iʃt/-exposed**			**Swabian speaker**		
CK (1786)	5957	5575	3770	6129	5815	3847	4672	6082	4258
DB (1733)	6690	6478	5063	6535	6740	5343	4553	5953	4206
AR (539)	6118	6284	3244	6036	6165	3976	5086	5890	4564
KS (489)	6226	6239	3810	6236	6337	4621	5073	6203	4536
ET (3555)	6184	5716	4136	6370	6078	4004	4378	5600	3954
TJ (2710)	5693	5526	3644	5959	5656	3223	4353	5787	3998
MK (576)	5779	5751	2989	6345	6210	3800	4330	6295	4099
Average	6092	5938	3808	6230	6143	4116	4635	5973	4231

Three linear-mixed effect models were run to test whether there is any alignment of fricative spectra in the data set. A first analysis established statistically that the interviewers and the Swabian speaker differed in their pronunciation of "ist". The dependent variable in this first analysis was the fricatives' CoG predicted by the fixed factors Fricative (three levels: "s", "sch", and "ist") and Role ("Interviewer" vs. "Interviewee") and their interaction. The level "s" for the Fricative factor and the level "Interviewer" for the factor Role were mapped on the intercept. To account for speaker and recording differences, a random intercept was added for each combination of speaker and recording, as well as a random slope for both Fricative and Role. Table 2 shows the resulting beta weights and their level of significance (based on the conservative assumption of 8 df, 14 independent observations minus 6 parameters). Going through Table 2, the Intercept value of 6145 reflects

[2]The German word *ist*/ɪst/ is often pronounced without the final /t/, and the resulting form can be subject to voice assimilation (*ist es*, Engl. 'is it', /ɪst#ɛs/ → [ɪzɛs]) as well as place assimilation (*ist schon*, Engl. 'is already', /ɪst#ʃon/ → [ɪʃ:on]).

[3]Words such as *demokratisch* (Engl., 'democratic') and bis (Engl., 'till') end on [ɪʃ] and [ɪs] respectively in both Standard and Swabian German.

the estimated mean for the combination of factor levels mapped on the intercept, which is the interviewers' average [s] CoG. The beta-weight for the Fricative level [ʃ] indicates that the CoG for [ʃ] by interviewers is more than 2000 Hz lower than their CoG for [s]. The insignificant beta-weight for the "ist" level of the factor Fricative shows that the interviewers produce a fricative in *ist* that is similar to their [s]. Note that the "main effect" for the Swabian interviewee is different from a main effect in an analysis of variance. In a regression model, it shows how the interviewee differs from the interviewers for the level [s] of the Fricative factor, which has been mapped on the intercept. As the estimate shows, there is no significant difference in the pronunciation of [s]. The interviewee produces, however, a slightly higher [ʃ], and, reflecting the Swabian accent, a massively lower fricative in *ist* than the interviewers.

Note that both interviewers and the Swabian speaker seem to produce a fricative in *ist* that is slightly higher than the "reference category" (i.e., /s/ for the interviewers and /ʃ/ for the Swabian speaker). This is likely a residual trace of the /t/ at the end of *ist*, which also has an alveolar place of articulation. Coarticulation with the /t/ would explain the slightly higher CoGs in ist compared to the relative reference category.

Table 2: Beta weights for the analysis comparing fricatives' center of gravity between interviewers and the Swabian speaker.

β	Estimate	t	p (based on df = 8)
Intercept	6145	45.1	< 0.001
Fricative = [ʃ]	-2025	-14.5	< 0.001
Fricative = "ist"	109	1.2	0.14
Role = Interviewee	-173	-1.1	0.16
Fricative = [ʃ] : Role = Interviewee	280	1.6	0.07
Fricative = "ist": Role = Interviewee	-1449	-9.3	< 0.001

Table 3: Beta weights for the analysis comparing interviewers' fricatives' center of gravity when the interviewee uses the Standard or Swabian variant.

β	Estimate	t	p (based on df = 8)
Intercept	6143	44.1	< 0.001
Fricative = [ʃ]	-2026	-13.7	< 0.001
Fricative = "ist"	117	-1.2	0.13
Interviewee = Standard	-210	-1.0	0.17
Fricative = [ʃ] : Interviewee = Standard	-90	-0.3	0.38
Fricative = "ist": Interviewee = Standard	54	0.4	0.34

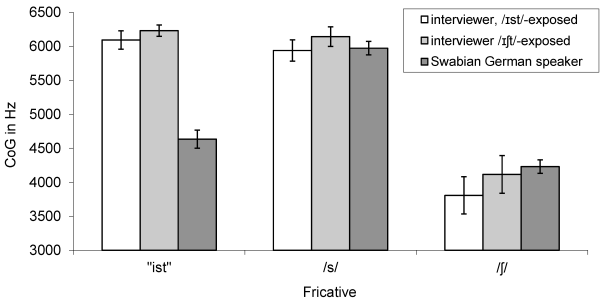

Figure 1: Overall mean spectral center of gravity for the fricatives [s], [ʃ], and the fricative in the German word *ist* (Engl.' is'). Note that centre of gravity of the fricatives in *ist* is similar to [ʃ] for the Swabian German speaker but that this does not influence the interviewers, who produce a fricative in *ist* that is close to their [s] independent of their interviewee's behavior.

Having established an overall difference between interviewers and their Swabian interviewee in the pronunciation of the fricative in *ist*, a next analysis tested whether interviewers produce different fricatives depending on the accent of their interviewee. Again, a linear mixed effect model was run with a random intercept plus random slopes for every combination of speaker and recording. The fixed factors were Fricative and Interviewee's Variant (Standard vs. Swabian) and their interaction. As Table 3 suggests, there was no measurable influence of the interviewees' variant on how the interviewer produces his/her fricatives. First of all, Figure 1 (comparing the white bars for the /ɪst/-exposed with the gray bars for the /ɪʃt/-exposed condition) shows that the observed CoGs in the /ɪst/-exposed condition are all slightly lower than in the /ɪʃt/-exposed condition. This is in all likelihood due to different recording set-ups and audio coding of the AV files for archiving, and is re-

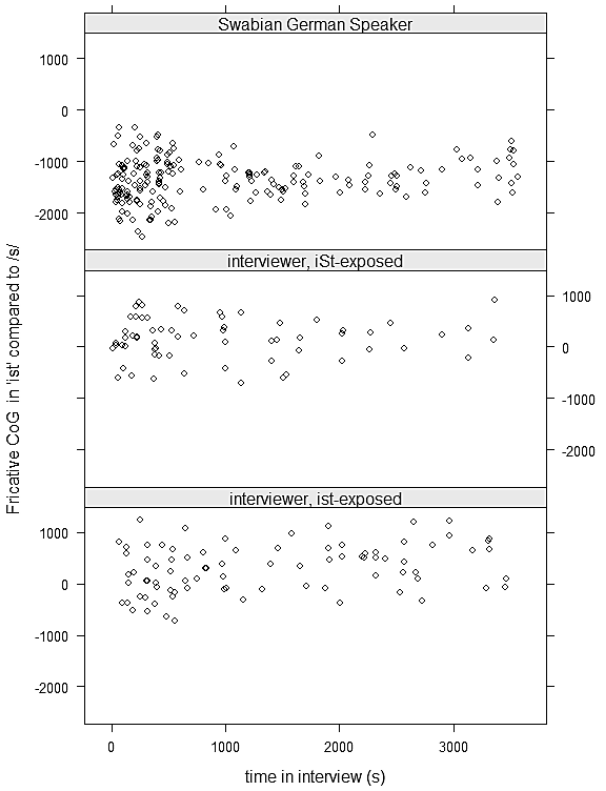

Figure 2: Individual fricatives' centre of gravity in the word *ist* (Engl. 'is', in comparison for the speaker and recording specific [s], plotted against interview duration. Phonetic alignment should lead to an upwards trend for the Swabian speaker and a downwards trend for interviewers when exposed to [ɪʃt] pronunciations. Such effects are neither visible nor found in the statistical analysis.

flected in the -210Hz, but not significant, beta-weight for "Interviewee = "standard". Relative to this baseline difference, the critical interaction term is Fricative = "ist" : Interviewee = "Standard", which indicates how much higher the fricative in ist is if the interviewer converses with a speaker that uses /s/ in *ist*. Note that the effect goes in the "expected" direction, but is, first, far from significant and, second, only about 1/30 of the difference between the speakers; the effect is 54 Hz compared to the nearly 1500Hz difference between interviewer and interviewee in the production of the fricative in *ist*. To confirm that this is no real effect, the initial model with the interaction was compared to the model without an interaction; an analysis that showed that the interaction did not explain any variance ($\chi^2(2) = 0.26$, $p > 0.2$).

Finally, it was tested whether there was any sign of alignment over the course of the interviews. To this end, we generated CoG values for the fricatives in *ist* which were normalized for speaker and recording influences by subtracting the mean [s] value for the recording/speaker combination in which a given token of *ist* occurred. The final regression model then tested whether these normalized values converged over time. The model predicted the normalized CoG of all *ist* tokens with the following fixed factors: Role with three levels ([ɪʃt]-exposed Interviewer, [ɪst]-exposed Interviewer, and Swabian German Interviewee) as well as time in interview. If there is convergence, the fricative CoGs should become higher over time for the interviewee and/or lower for the interviewers when [ʃt]-exposed. The first effect would show that the interviewee aligns with the interviewer while the latter would show that the interviewer aligns with the interviewee. As Figure 2—with all data points for this analysis—suggests, the patterns were stable over time. The beta-weights of the linear mixed effect model shown in Table 4 confirm this. There is no significant interaction of Time and Role. In fact, a regression model with only Role as predictor does not explain less variance than a model with Time and its interaction with Role ($\chi^2(3) = 1.32$, $p > 0.2$). This indicates that the speakers were stable over time.

Table 4: Beta weights for the analysis testing an influence of time on the normalized fricatives' center of gravity in the German word *ist* ('is) for standard speakers under different exposure condition and the divergent Swabian speaker.

β	Estimate	t	p (based on df = 8)
Intercept	207	2.1	0.03
normalized Time	-1	-0.4	0.65
Role = iSt-exposed interviewer	-43	-0.3	0.35
Role = Swabian Interviewee	-1589	-10.7	< 0.001
normalized Time : iSt-exposed Interviewer	-1	-0.2	0.42
normalized Time : Swabian Interviewee	1	0.7	0.24

4 Discussion

The current result indicates that speakers with different accents can maintain their phonetic differences over the course of a conversation. This finding has several theoretical consequences. Phonetic alignment is often portrayed as an automatic consequence of being in a dialogue, with social influences only moderating the inevitable alignment (Miller et al., 2010). The current data set shows that alignment of speech gestures for a

given segment is not inevitable and can be avoided completely.

The contrast of the current study (with no alignment) and other studies (finding alignment at least in perceptual measures), raises the question which parameters are likely to give rise to alignment. First of all, the difference in pronunciation of *ist* in German is well represented in the public conscience, possibly because the difference can be coded orthographically. It might hence be that being conscious of a difference impedes alignment. However, other studies using both acoustic measures and perceptual judgments (Babel et al., 2013; Pardo et al., 2013) also find little alignment of segmental properties such as vowel spectra, which are difficult to capture in orthography, but still find clearer effects in perceptual judgments. These are probably driven by prosodic properties. It may hence be the case that prosodic parameters are more likely to give rise to alignment than segmental properties.

Finding that alignment might not be a consequence of a direct perception-action link suggests that social variables may not be moderators but actually the driving forces of alignment. A similar conclusion is reached by a study (Gregory and Webster, 1996) that analyzed a database of interviews from *Larry King Live*. They evaluated the average spectrum in the band 0-0.5 kHz of different interviewees and Larry King. Based on correlations between different spectra, they argued that interviewer and interviewee accommodate to one another and that who accommodates to who is dependent on the relative social status. Much of their findings, however, are questionable because the analysis was built on wrong assumptions about what drives correlations between spectra[4].

It has also been suggested that alignment fosters mutual understanding (e.g., (Miller et al., 2010; Pickering and Garrod, 2004)). While it is difficult and probably impossible to judge the quality of an interview, listening to the interviews while searching for tokens of *ist* did not suggest that interviewers had trouble in spoken-word recognition caused by the different pronunciation of *-st* clusters by the Swabian interviewee. The current literature on spoken-word recognition indeed suggests that listeners can adapt fast to speaker-specific idiosyncrasies (Clarke and Garrett, 2004; Eisner and McQueen, 2006; Kraljic and Samuel, 2006; Maye et al., 2008; Norris et al., 2003). Quite relevant for the current purposes, (Kraljic et al., 2008) tested adaptation to variation in the pronunciation of fricatives as either /s/ or /ʃ/. They found that quick adaptation in perception did not have any repercussions for production. Similarly, a study by (Mitterer and Ernestus, 2008) suggests that a difference in speech production patterns does not have to hinder perception. In this study, participants had to shadow /r/-initial nonwords in Dutch. The initial /r/ was produced as either an alveolar or a uvular trill, with both variants being common in the Netherlands. Participants did not imitate the variation in the trill; an unsurprising finding as most Dutch speakers master only one trill variant. More interestingly, however, the shadowing latencies were not slowed down by the consequential gestural mismatch between stimulus and response. Participants were just as fast in producing a nonword when the stimulus contained their preferred trill than when it contained the other trill. These datasets seem to converge on the conclusion that a divergence in phonetic patterns does not necessarily impede speech perception and spoken-word recognition. Two interlocutors can agree to disagree on how to produce certain words without negative consequence for mutual understanding.

[4]These authors assume that neither the speaker intrinsic f0 range nor the vocal effort influence the correlation between two spectra. However, a speaker with a average f0 of 130 Hz will have "bumps" in the spectra at the harmonics of the modal f0 which influence the shape of the spectrum. Increasing vocal effort does not only influence the average level of a spectrum, but also changes the spectral tilt and thereby also influence the shape of a spectrum.

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Review Article

Pharmacogenetics: the science of predictive clinical pharmacology

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Abstract. The study of pharmacogenetics has expanded from what were initially casual family-based clinical drug response observations, to a fully-fledged science with direct therapeutic applications, all within a time-span of less than 60 years. A wide spectrum of polymorphisms, located within several genes, are now recognised to influence the pharmacokinetics and pharmacodynamics of the majority of drugs within our therapeutic armamentarium. This information forms the basis for the new development of pharmacogenetic genotyping tests, which can be used to predict the therapeutic and/or adverse effects of a specific drug in a particular patient. Pharmacogenetic-guided, patient targeted therapy has now become the developing fulcrum of personalized medicine, as it provides the best means to optimize benefit/risk ratio in pharmacological management.

Keywords Pharmacogenetics - targeted therapy - predictive biomarkers - personalized medicine.

1 Introduction

The understanding of molecular pathology of disease, together with the discovery of novel therapeutic targets, has greatly enhanced the evolution of new drugs. On the forefront of such discoveries lie the various biotech drugs produced by recombinant DNA technology, such as the anti-cancer drugs trastuzumab and ibrutinib; the latter being granted a license by the FDA in February 2014 (FDA, 2014); as well as recent receptor-specific ligands such as the angiotensin II receptor blocker azilsartan, which was approved by the FDA for use in hypertension management in February 2011 (FDA, 2011). Although scientific knowledge in this area has been increasing exponentially, it has frequently been observed that different patients often exhibit altered responses to such therapies. These responses may manifest as very high or very low drug efficacy, or as an unexpected adverse reaction in selected individuals. Genetic variation is a common contributor to altered responses. Pharmacogenetics, a term first coined by Friedrich Vogel in 1959 (Vogel, 1959), is the study of the association between genetic variation (most commonly single-nucleotide polymorphisms (SNPs) or microdeletions), and the efficacy and toxicity of a drug.

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2 Pharmacogenetic biomarkers

Pharmacogenetics more commonly deals with the identification of germ-line genetic variants as predictive markers of drug response. However, in some patient groups, such as those suffering from cancer, genomic analysis is possible both on germ-line DNA, often derived from blood, as well as on somatic DNA, derived from tumour tissue (biopsies or resections). Whereas germ-line DNA variants are inherited in normal Mendelian fashion, somatic DNA mutations represent the variations associated with tumour initialisation and progression, or secondary mutations due to the tumour itself (Gerlinger et al., 2012). For example, genetic variations in the genes that code for drug metabolising enzymes, such as thiopurine-S-methyltransferase (TPMT), cytochrome P450 2D6 (CYP2D6) and uridine-diphosphate glucuronosyltransferase 1A1 (UGT1A1) are germ-line polymorphisms useful for determining pharmacokinetically-influenced pharmacological outcomes. Table 1 summarises a few examples of such pharmacogenetic variability. On the other hand, acquired somatic mutations in tumour tissue may regularly directly modify the pharmacodynamics of drug response. Somatic mu-

tations are exemplified by the presence of the BRC-ABL (a reciprocal chromosomal translocation between chromosomes 9 and 22, resulting in the generation of a fusion gene, consisting of the chromosome 9 ABL1 gene and part of the BCR gene on chromosome 22) translocation in chronic myeloid leukaemia (CML), the overexpression of human Epidermal Growth Factor Receptor 2 (HER2) in breast cancer, BRAF (a gene coding for serine/threonine-protein kinase B-Raf involved in cell-growth signalling) mutations in metastatic melanoma, and Epidermal Growth Factor Receptor (EGFR) mutants in lung cancer. The abnormal functioning of these oncogenic proteins promotes cell transformation. The transformed cells become dependent on the oncogenic stimulus and hence provide a therapeutic opportunity, due to the increased sensitivity of the transformed cells to specific inhibitors. This is the basis of targeted therapies (Weinstein, 2008). The tissue-specific variations are classified as prognostic or predictive biomarkers. A prognostic biomarker is defined as a measurable disease-related parameter that is associated with clinical outcome. On the other hand, a predictive biomarker provides information on the expected response to a specific therapy, therefore identifying patients who are responsive to a specific treatment, and/or predisposed to toxicity. Interestingly, BRAF mutations provide prognostic information in colorectal cancer, but they are predictive in melanoma (Chapman et al., 2011).

3 Genetic influences on drug pharmacokinetics

The administration of a drug is followed by multiple processes, which comprise the pharmacodynamic events through which its effects manifest themselves; and pharmacokinetic events, through which the drug becomes subject to modification by physiological systems. Such pharmacokinetic events may include bioactivation to the active form, as well as various detoxification processes, which occur prior to elimination. A group of enzymes, the Cytochrome P450 (CYP450) family, are the major contributors to drug activation and metabolic processes (Guengerich, 2008; Ortiz de Montellano, 2013). CYP450 genes are highly polymorphic, resulting in variable activity of enzymes and hence variable processing of the drug within human physiology. Of interest, the polymorphic nature of these enzymes is even more pronounced across different ethnic groups (McGraw and Waller, 2012). The Human Cytochrome P450 Allele Nomenclature Committee maintain a public database, that details all known CYP450 gene alleles and the influence of these alleles on specific CYP450 enzyme activities (Human Cytochrome P450 (CYP) Allele Nomenclature Committee., 2013). Examples of CYP2D6 functional variants are discussed further below.

3.1 Biotransformation of prodrugs

The development of prodrugs provides the chemical approach to overcome the therapeutic barriers of some drugs, such as mal-absorption, inadequate distribution to specific destinations in the body and premature detoxification, which may, for example, occur during the hepatic first pass effect. Prodrugs are administered as pharmacologically inactive or low activity compounds and require prior conversion to the pharmacologically active metabolite in order to function. The exploitation of endogenous enzymatic systems for the bioactivation of prodrugs may be influenced by genetic variations within populations and ethnic groups that are associated with an increased, reduced or even no enzymatic activity.

3.1.1 Dopa decarboxylase

One of the best known pro-drug applications has been the development of L-Dopa for the management of Parkinson's Disease (PD). L-Dopa crosses the blood brain barrier, and is metabolized to dopamine in the brain by aromatic L-amino acid decarboxylase (commonly called DOPA decarboxylase, DDC). Directly administered dopamine would be unable to cross the blood brain barrier and would exert several peripheral adverse effects, making it unsuitable for management of PD. The actions of L-dopa-generated dopamine are further centralized to the brain by the co-administration of carbidopa, a peripheral dopamine decarboxylase inhibitor, which effectively minimizes peripheral metabolism of L-dopa to dopamine. Alterations in the function or expression of DDC may therefore be expected to influence L-Dopa treated PD management outcomes. Indeed, two DDC gene variants (rs921451 - an intronic T>C substitution, and rs3837091 - a four-base AGAG deletion in the non-translated exon 1) that show significant decreases in L-dopa treated PD patient management outcomes have recently been reported (Devos et al., 2014).

3.1.2 CYP2D6

Within the family of CYP450 enzymes, CYP2D6 is perhaps the most pharmacogenetically-relevant member. This is in view of its participation in the metabolic pathways of multiple drugs, originating from different pharmacological classes (e.g. selective serotonin reuptake inhibitors, opioids, tricyclic antidepressants, β-adrenergic antagonists, antipsychotics, antiarrhythmics), and the highly polymorphic nature of the gene. The enzyme is important both in lieu of its drug metabolizing and detoxification properties (discussed separately further down), as well as a participant in the metabolic activation of some prodrugs.

Table 1: A non-exhaustive list of genes, for which known alleles influence the outcomes of specific drug therapy.

Gene	Drug(s) affected	Outcome	Reference
ABCB1	Various	Variants causing ABCB1 overexpression increase cellular efflux and reduce activity of affected intracellular-acting drugs. Variants causing low ABCB1 expression tend to increase adverse effects due to low efflux and intracellular drug accumulation.	(Franke et al., 2010)
OATP1B1	rosuvastatin	OATP1B1*5 homozygotes exhibit higher plasma levels of rosuvastatin	(Choi et al., 2008)
SLCO1B1	simvastatin	Increased risk of myopathy in patients with the rs4149056 C-variant	(Brunham et al., 2012)
SLCO1B1	simvastatin	Variant rs4149056 associated with myopathy in patients on 80mg daily simvastatin	(Carr et al., 2013)
UGT1A1	irinotecan	UGT1A1*28 allele causes low drug metabolism and potential life threatening effects including myelosuppression, arrhythmia, neutropenia, thrombocytopenia and diarrhoea	(Dias et al., 2012)
G6PD	dapsone	G6PD deficient individuals may be at an increased risk of haemolytic adverse reactions	(Mason et al., 2007)
G6PD	nitrofurantoin	G6PD deficient individuals may be at an increased risk of haemolytic adverse reactions	(Youngster et al., 2010)
VKORC1	warfarin	G-1639A allele causes increased bleeding risk especially when present with CYP2C9*2 or CYP2C9*3 alleles	(Santos et al., 2013; Yang et al., 2013)
POLG	valproic acid	Increased risk of potentially fatal valproate-induced acute liver failure, especially in patients with the Q1236H substitution.	(Stewart et al., 2010)
NAT2	isoniazid, rifampicin	NAT2 slow acetylator genotypes *5, *6 and *7 are associated with higher risk of anti-tuberculosis drug-induced liver injury	(Wang et al., 2012)
CYP2C9	warfarin	CYP2C9*3 homozygotes exhibit an extended warfarin half-life with possible haemorrhagic events	(Santos et al., 2013; Yang et al., 2013)
CYP2D6	codeine	CYP2D6 ultrarapid metabolizers may show opioid toxicity due to extensive metabolism of codeine to morphine	(Kirchheiner et al., 2007)
CYP2D6	amitriptyline, nortryptiline	CYP2D6 ultrarapid metabolizers experience no response, while poor metabolizers experience drug toxicity at conventional dosages	(Teh and Bertilsson, 2012)
CYP2D6	tamoxifen	CYP2D6*10 homozygotes experience poor response to tamoxifen	(Kiyotani et al., 2012)
DDC	L-dopa	DDC rs921451 and rs3837091 variants are associated with poor L-dopa-treated Parkinson's disease management outcomes	(Devos et al., 2014)
TPMT	azathioprine	TPMT*2, TPMT*3 or TPMT*4 alleles have a low TPMT activity, and patients show azathioprine toxicity	(Ford and Berg, 2010; Relling et al., 2011)
UGT1A1	irinotecan	Variant *28 associated with high risk of neutropenia	(Perera et al., 2008)
ALOX5	ABT-761, zileuton	Patients carrying non-pentarepeat Sp1 promoter variants, express low ALOX5, and show reduced response to 5-LOX inhibitors	(Drazen et al., 1999)
ADRB2	carvedilol	CHF patients who are Gln27 homozygotes show lower response to treatment than Glu27 homozygotes or heterozygotes.	(Kaye et al., 2003)

ADRB2	salbutamol salmeterol	Variant Gly16 associated with lower response to β_2-adrenoceptor agonists	(Hall et al., 1995; Lipworth et al., 2013)
OPRM1	morphine and other opioids	A118G allele associated with reduced surface receptor expression and low response to exogenous opioids	(Zhang et al., 2005; Kroslak et al., 2007)
NR3C1	glucocorticoids	Variants Ala229Thr and Ile292Val associated with decreased receptor ligand binding affinity; variant T746C and haplotype 237delC/C238T/G240C associated with lower glucocorticoid receptor expression.	(Niu et al., 2009)
NR3C1	glucocorticoids	Glucocorticoid receptor promoter BclI G allele associated with glucocorticoid resistance	(Pietras et al., 2011)

Within the latter perspective, the CYP2D6 gene was extensively studied within the context of tamoxifen metabolism. Tamoxifen is used to treat oestrogen receptor positive breast cancer patients, and is metabolised to endoxifen, a more potent molecule, by CYP2D6. The wild type CYP2D6 (CYP2D6*1) generates an enzyme with normal activity. The variants *3, *4 and *5 have null activity and the variants *9, *10 and *17 have a decreased enzyme activity. Compared to extensive metabolisers (EMs) having both wild type alleles, patients with one null allele, or with one or more decreased activity alleles have an 'intermediate metabolism' (IM) phenotype, whilst patients carrying two null alleles are 'poor metabolisers' (PM). This variation carries therapeutic implications in patient tamoxifen sensitivity (Crews et al., 2012).

Codeine, an opiate agonist indicated for the relief of mild to moderately severe pain, is converted to an active metabolite, morphine, via the CYP2D6 enzyme, in order to exert its analgesic activity. The analgesic activity of codeine is therefore due to the combined action of the weak parent molecule and the potent CPY2D6-dependent metabolite, morphine; thus making codeine a prodrug. The presence of a CYP2D6 allele, which generate a low activity enzyme (poor metabolisers, such as individuals carrying CYP2D6*4, *5, *6, or *7 alleles) has been associated with a reduced codeine response, while high CYP2D6 activity in ultrarapid metabolisers (due to CYP2D6 gene copy number variation), have been reported to induce morphine toxicity with conventional codeine doses (Gasche et al., 2004).

CYP2D6 alleles, which mainly consist of haplotypes rather than single SNPs, have been classified by The Human Cytochrome P450 Allele Nomenclature Committee, and the specific genotype of each allele is archived at an official database hosted at http://www.cypalleles.ki.se/cyp2d6.htm.

3.1.3 Carboxylesterases

Carboxylesterases represent a class of prodrug bioactivating enzymes that are widely distributed in tissues including plasma, liver, intestine and other biological fluids (Hosokawa, 2008). They metabolize prodrugs, such as olmesartan medoxomil (an angiotensin II receptor antagonist) and oseltamivir (an antiviral agent) to their respective pharmacologically active metabolites olmesartan (Ishizuka et al., 2010) and oseltamivir carboxylate (Zhu and Markowitz, 2009). The identified carboxylesterase 1 variant Gly143Glu, has been reported to reduce the activity of the prodrug-activating enzyme to 25% of the wild type, and the Asp260fs variant ablates activity completely. This may have serious implications, with respect to the efficacy of oseltamavir in patients carrying these variants (Zhu and Markowitz, 2009).

The inhaled glucocorticoids beclomethasone and ciclesonide are also activated by lung esterases. Inhaled beclomethasone diproprionate is metabolised to the more active beclomethasone-17-monopropionate, while ciclesonide, an inactive prodrug, is metabolised in the lungs to the pharmacologically active desisobutyryl-ciclesonide. Although genetic variability in various esterase genes has been described (Wu et al., 2004; Charasson et al., 2004), pharmacogenetic evidence for their relevance to bioactivation-associated pharmacology is lacking. There is evidence, however, to indicate pharmacogenetic importance of these enzyme systems as detoxifying agents in the metabolism of active drug molecules such as methylphenidate (Zhu et al., 2008), where specific SNPs have been shown to drastically alter the rate of metabolism.

3.2 Membrane transporters

Drug transport across cell membranes may occur either by passive mechanisms, or through the mediation of specific cell-membrane proteins which actively transfer the drug molecule between the extracellular environment and the intracellular milieu. More than 400 membrane transporters are known to be coded for by the human genome, and these are differentially expressed in various tissues. Genetic variability in the two major eukaryotic transporter families, the ATP-binding cassette (ABC) and Solute Carriers (SLC), is recognised to have major influences on drug therapeutic efficacy and adverse reactions. Such changes may

be brought about by functional polymorphic variation which influences either the eukaryotic ABC-mediated efflux functions, or the SLC uptake efficiency, or both. A database of known transporters may be accessed at the Human Membrane Transporter Database, available at http://lab.digibench.net/transporter/ (Yan and Sadée, 2000).

3.2.1 ATP-binding cassette (ABC) transporter family

P-glycoprotein (Pgp)

P-glycoprotein (Pgp), a major transporter of the ABC family, is encoded for by the ABCB1 gene (previously known as MDR1) and mediates the ATP-dependent efflux of drugs from cells. It is expressed in various tissues including intestinal, hepatic and renal. It also contributes to the maintenance of the blood-brain barrier system through endothelial expression in the central nervous system. Pgp substrates originate from diverse pharmacological classes, and include immunosuppresants, cardiovascular agents, antidepressants and anti-epileptic agents, as well as cytotoxic drugs. ABCB1 overexpression is a major concern in cancer chemotherapy since it induces resistance to cytotoxic agents through increased cellular efflux. For example, high-expression inducing ABCB1 G2677T and G2995A SNPs have been identified in tumour patients, in drug-resistant cell lines (e.g. colon cancer cell lines and glioblastoma cell lines) and also in cells from refractory malignant malignomas. ABCB1-knockout mice show increased tendency toward dose-related adverse drug reactions, increased blood brain barrier transport and a degree of altered pharmacokinetics to a plethora of drugs including paclitaxel, loperamide, vinblastine, ivermectin, digoxin and cyclosporine. This is accompanied by drug accumulation in the brain, liver and intestine.

The ABCB1 C3435T SNP is associated with low intestinal Pgp expression, especially in homozygotes, and these comprise about 25% of Caucasians. The reduced intestinal cellular Pgp efflux activity in these individuals has been associated with increased plasma levels of administered digoxin, due to increased uptake of the drug from the gastrointestinal tract. There are about 20 known human ABCB1 variants to date, and their functional relevance is still coming to light. The differential expression of this gene and its recognised genetic polymorphic profile makes it an important potential target for altered pharmacokinetic behaviour of drug substrates, and the subsequent pharmacodynamic implications. For example, high Pgp activity induces cellular efflux of glucocorticoids such as prednisolone, dexamethasone and beclomethasone monopropionate (the active metabolite of the pulmonary administered beclomethasone dipropionate), thus decreasing their clinical response. There is also evidence to show that glucocorticoids upregulate Pgp expression and may therefore amplify the effects of ABCB1 allele-specific high activity variants (Crowe and Tan, 2012).

3.2.2 Solute Carrier (SLC) transporter family

Organic anion-transporting polypeptides (OATP)

Organic Anion-Transporting Polypeptides (OATP), the major SLC-type transporters, have received special attention due to their involvement in the cellular influx of several drugs, particularly statins (HMG-CoA reductase inhibitors). OATPs are highly expressed in hepatic tissue, where they are involved in the clearance of drugs from portal circulation in preparation for subsequent biliary excretion. The influx transporter OATP1B1 (coded for by the SLCO1B1 gene) has, in particular, been well studied due to its major role in the hepatic uptake of many drugs. For example, the SLCO1B1 alleles *2 (T217C), *3 (T245C, A467G), *5 (T521C), *9 (G1463C), *12 (T217C, A1964G), *13 (T245C, A467G, A2000G), *15 (A388G, T521C), and *18 (A388G, G1463C) have all been associated with decreased rosuvastatin transport activity. SLCO1B1*15 homozygotes in particular have been reported to exhibit significantly higher AUC values for concentration-time curves of plasma rosuvastatin, possibly due to decreased intracellular drug influx (Choi et al., 2008).

Na^+-taurocholate cotransporting polypeptide (NTCP)

A variant of another SLC member, the Na^+-taurocholate cotransporting polypeptide *2 allele (NTCP *2, C800T), is known to have a near complete loss of function for bile acids; however it exhibits a profound gain of function for rosuvastatin, resulting in a clinically relevant reduction in plasma levels compared to the wild-type allele. The outcome may be further complicated by the contribution of OATPs such as OATP1B3, OATP2B1 and OATP1A2, which are also known to mediate statin transport and exhibit functional polymorphic variation in humans (Choi et al., 2011).

Organic anion transporter family member 1B1 (SLCO1B1)

The solute carrier organic anion transporter family member 1B1 (coded for by SLCO1B1) is expressed and localised in the cell membrane of hepatocytes and is involved in uptake of xenobiotic and endogenous substances (Karlgren et al., 2012). During the recent years, the SLCO1B1 SNP T37041C (rs4149056) began to receive special attention following the development of myopathy in patients on high dose simvastatin (80mg daily) participating in the 2008 SEARCH case-control study, and who were later recognised to carry the minor

C allele. The localisation of the transporter in the hepatic plasma membrane is disturbed in the presence of this variation, resulting in a higher simvastatin plasma concentration and increased skeletal muscle drug exposure (Pasanen et al., 2006). The FDA strongly advises against 80mg daily simvastatin doses for patients who are heterozygous or homozygous for the C allele. The FDA also advises caution for long term use of 80mg daily doses in wild type 37041T homozygotes. These recommendations have also been incorporated into the SLCO1B1 Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines (Wilke et al., 2012).

3.3 Drug metabolism

Drug metabolism is a complex process, often involving multiple parallel pathways, during which drugs are converted to breakdown products (usually of lower toxicity) in preparation for elimination from the physiological system. The rate of drug breakdown is important in reducing toxicity level and maintaining a pharmacological dose within the therapeutic window of the active ingredients.

3.4 Thiopurinemethyltransferase (TPMT)

Thiopurinemethyltransferase (TPMT) is a phase II biotransformation cytosolic enzyme catalysing the methylation of thiopurines into inactive metabolites. The TPMT enzyme is highly expressed in the liver, while its expression is low in the brain and lung (Ford and Berg, 2010). Interestingly, the TPMT activity in erythrocytes correlates with the hepatic enzyme activity, meaning that phenotypic assessment can be performed in erythrocyte lysates (Wu, 2011). The TPMT gene is polymorphic and despite ethnic variability, the minor alleles TPMT*2, TPMT*3A and TPMT*3C account for the majority of the low-activity variants (Relling et al., 2011). TPMT activity shows a trimodal distribution with heterozygotes having intermediate activity (IM), homozygotes or compound heterozygosity showing low or absent activity (PM), and the normal genotype TPMT*1/*1 representing the normal activity of the enzyme (EM). The major drug for which TPMT activity is relevant is mercaptopurine, the active metabolite of the prodrug azathioprine. Mercaptopurine is detoxificated by several metabolic pathways, including a major one that involves the enzyme TPMT. Azathioprine patients with low TPMT activity are at a strong risk of developing severe, potentially life-threatening, bone marrow toxicity when treated with conventional doses of azathioprine or mercaptopurine (Anon, 2009; Ford and Berg, 2010).

3.5 UDP Glucuronosyltransferase 1 (UGT1A1)

Irinotecan, a topoisomerase I inhibitor used to treat several solid tumour types, as well as its active metabolite SN-38, exert their effects by preventing re-ligation of single-stranded DNA breaks induced during the DNA synthesis phase of cellular replication. SN-38 subsequently undergoes glucuronidation primarily in the liver by UGT1A, followed by excretion through the kidneys.

Adverse effects of irinotecan treatment include severe diarrhoea, myelosuppression, and neutropenia. These effects are induced by inefficient UGT1A1-dependent metabolism of SN 38 (Mathijssen et al., 2001). UGT1A1 gene variants, which demonstrate a reduced enzyme activity or expression, cause an accumulation of the active metabolite SN-38 at conventional urinotecan dosing. Of primary importance is the high frequency variant UGT1A1*28, which results in at least 70% reduction in expression levels (Perera et al., 2008). Cancer patients homozygous for the *28 allele, receiving irinotecan at 350 mg/m^2 every 3 weeks, have a high risk of suffering from neutropenia. Genotype-guided phase I studies determined the maximum tolerated dose in *1/*1, *1/*28, and *28/*28 patients to be 390, 340, and 150 mg/m^2, respectively (Marcuello et al., 2011). Indeed, the FDA recommends an initial dose reduction of irinotecan for patients who are homozygous for UGT1A1*28, this is supported by the CPIC and Dutch Pharmacogenetics Working Group (DPWG) guidelines, which recommend a 30% dose reduction in irinotecan administration for this genotype (Relling and Klein, 2011).

3.6 Uridine diphosphate glucuronosyltransferase 2B7 (UGT2B7)

The UGT2B7 gene is highly expressed in the liver, and is pharmacogenetically relevant due to its importance in the detoxification of morphine. The gene product converts the drug to the inactive form morphine-3-glucuronide and the low efficacy morphine-6-glucuronide metabolites. Two tightly linked UGT2B7A polymorphisms, the 161C/T promoter SNP and the 802C/T SNP, are associated with low glucuronidation of morphine, with consequent clinical implications in opioid efficacy (Sawyer et al., 2003; Eissing et al., 2012).

3.7 Cytochrome P450 enzyme system (CYP450)

Some examples involving CYP450 members concerned in prodrug activation have already been cited earlier in this article. However, the metabolic detoxification role of these enzymes has more widespread implications, and has been much more thoroughly studied. Indeed, this enzyme system has been reported to partake in over 75% of drug detoxification processes that occur in man.

CYP2D6

The CYP450 member, which is most relevant to drug metabolism processes is the D6 enzyme of the CYP450 family 2 (CYP2D6). This enzyme is capable of metabolizing drug substrates that originate from chemically and pharmacologically distinct groups, including antidepressants, antipsychotics, antiarrhythmic, anti-emetics, beta-blockers and opioids. It is estimated that about 25% of currently marketed drugs have their metabolism in some way influenced by CYP2D6 activity, with about 100 commonly used drugs being major substrates for the enzyme.

The CYP2D6 gene is highly polymorphic. The Human Cytochrome P450 (CYP) Allele Nomenclature Committee currently lists over 100 known CYP2D6 genotypes, which may result in the expression of a normal or altered-activity CYP2D6 enzyme (http://www.cypalleles.ki.se/cyp2d6.htm). According to the specific allele combination carried, individuals may be classified as poor, intermediate or extensive metabolisers. Moreover, copy number variations of the CYP2D6 gene have also been described in the literature (Sheng et al., 2007), leading to the ultrarapid metaboliser phenotype. It has been estimated that as much as twenty million inhabitants of Western Europe are ultrarapid metabolisers, with at least a copy number of 2 functional CYP2D6 alleles (Ingelman-Sundberg, 2005). Extensive and ultrarapid metabolisers may show insufficient or no clinical response to CYP2D6 enzyme substrate drugs used at standard conventional dosages, while poor metabolisers run the risk of drug accumulation carrying consequent adverse reactions. For example, adverse effects to metoprolol, nortryptiline, perphenazine, thioridazine, haloperidol and perhexiline have been reported to be much more common in poor CYP2D6 enzyme metabolisers, while extensive metabolisers tend to show reduced effects (Teh and Bertilsson, 2012). Post-menopausal oestrogen receptor positive breast cancer patients on tamoxifen, who are also CYP2D6*4 homozygotes, show a significantly reduced relapse-free survival period when compared to wild type patients. Studies in Asian and Dutch populations have reported similar low tamoxifen response in breast cancer patients who are CYP2D6*10 homozygotes. It has been suggested that an increase of 50 to 100% of the normal tamoxifen dose is required for patients who are heterozygous or homozygous for the CYP2D6*10 allele, in order to maintain normal blood levels (Kiyotani et al., 2012).

The importance of CYP2D6 pharmacogenetics is further accentuated by the fact that the allelic frequencies of this gene are well known to exhibit diverse ethnic discrepancies, and alleles which may be present in one population or ethnic group, may be completely absent in another. This adds complexity to the genotype-phenotype interpretations.

CYP2C9

CYP2C9 gene polymorphic variability has been reported to be of pharmacogenetic relevance in the metabolism of warfarin (a commonly used anticoagulant) and celecoxib (a cyclooxygenase 2-specific non-steroidal anti-inflammatory drug). The nonsynonymous polymorphisms CYP2C9*2 (rs1799853) and CYP2C9*3 (rs1057910) express a reduced activity variant of the enzyme. CYP2C9*3 homozygotes may exhibit a warfarin half-life of 90-200 hours, thus potentially leading to serious warfarin-induced haemorrhagic events (Jorgensen et al., 2012). The same CYP2C9*3 genotype also increases the half-life of celecoxib by about 2.7 fold, and *3 homozygotes therefore warrant lower doses of the drug in order to avoid dose-related adverse effects (Prieto-Pérez et al., 2013).

4 Genetic influences on drug pharmacodynamics

4.1 Cell signalling

About seventy percent of drugs licensed for human therapeutic use mediate their action through their interaction with one or more cellular receptor proteins. Two thirds of these are dependent on one or more members of the G-protein coupled receptor superfamily. The rest act via nuclear or kinase receptors. Drug receptors, therefore, often constitute the first interface between a therapeutically administered drug and cellular physiology. Genetic variability in the receptors themselves, or any proteins involved in receptor signalling pathways, may exert a potential influence on therapeutic outcomes.

4.1.1 G-protein coupled receptors (GPCRs)

Genome sequencing data suggests that the human genome codes for about 800 different GPCRs. Nearly 400 of these are currently indexed in the International Union of Basic and Clinical Pharmacology online database (IUPHAR database, 2013), together with additional data such as: known ligands, gene and protein records and signalling pathways. The intricate signalling and trafficking pathways of GPCRs provide several targets within which genetic variability may influence drug efficacy, potency and safety. Besides the commonly reported ligand binding and subsequent signalling pathways, GPCRs may also show constitutive activity and may undergo other processes such as: desensitization, supersensitization, downregulation, upregulation, homodimerization, heterodimerization, trafficking and recycling (Bosier and Hermans, 2007;

Thompson et al., 2008b; Thompson et al., 2008a).

The most common loci that are known to cause perturbation of GPCR function however, remain to be those within the actual receptor proteins and G-protein complexes, as well as their respective gene promoters. We present some examples of pharmacogenetically relevant GPCR variants.

β_2-adrenoceptor

One of the most pharmacogenetically studied GPCRs is the β_2-adrenoceptor, coded for by the ADRB2 gene located at 5q31-q32. Polymorphic variations in this gene have been associated with: (i) perturbations of molecular pharmacological actions, such as alterations in downregulation activity, (ii) changes in therapeutic outcomes of β_2-adrenoceptor agonist treatment and (iii) changes in clinical manifestations of different diseases. For example, the widely studied Arg16Gly receptor variant displays enhanced agonist-promoted downregulation, while the Gln27Glu polymorphism appears to confer resistance to downregulation. Patients with asthma carrying the Arg16Gly β_2-adrenoceptor variant, have been reported by some research groups to exhibit a lower lung function than Arg16 patients, and also show an increased incidence of familial nocturnal asthma. These patients have also been reported to be less responsive to the commonly used β_2-adrenoceptor agonist bronchodilator treatment, probably due to reduced cell surface density of β_2-adrenoceptors. However, other clinical studies in asthmatic patients have reported ADRB2 genotype-phenotype associations in small-scale adult clinical studies, that conflict with earlier findings (Bleecker et al., 2007), and research workers have argued that sample size, patient selection, and concomitant treatment could confound the study outcomes. (Hall et al., 1995; Tan et al., 1997; D'Amato et al., 1998; Lee et al., 2004; Tattersfield and Hall, 2004). Recently, (Lipworth et al., 2013) studied 62 persistent asthmatic children who were homozygous for the ADRB2 Arg16 polymorphism and were being treated with regular inhaled fluticasone. In these Arg16 homozygotes, add-on therapy, with the leukotriene receptor antagonist montelukast, produced significantly greater clinical improvements than the long acting β_2 adrenoceptor agonist salmeterol when assessed after one year. The authors suggest that these preliminary data indicate that genotyping of the ADRB2 codon 16 may be a useful pharmacogenetic marker, which could have potential to be used to optimize asthma management (Lipworth et al., 2013; Sayers, 2013).

Leukotriene receptors

Leukotrienes constitute an important group of pro-inflammatory and bronchoconstrictory molecules, derived via the 5-lipoxygenase-mediated (5-LOX) arachidonic acid metabolism pathway. The major cysteinyl leukotrienes comprise LTC4, LTD4 and LTE4, while LTB4 which lacks cysteine, is classified separately. The former exert their actions via agonism at the the cysteinyl leukotriene receptor CysLTR1 (and to a lesser degree via CysLTR2), while the latter exerts its actions via LTB4R and LTB4R2. Antagonism of the CysLTR1 receptor by drugs such as zafirkulast, montelukast and pranlukast is an important therapeutic modality in the management of asthma and allergic rhinitis. Although genetic CysLTR1 variability is well documented, functional pharmacological effects have mainly been observed when CYSLTR1 SNPs are present together with SNPs in the 5-lipoxygenase gene ALOX5. For example, ALOX5 promoter variants, involving polymorphisms which result in an alteration of the number of Sp1 binding motifs, from the wild-type penta-Sp1 repeat, are known to result in low 5-lipoxygenase expression. This results in a reduced contribution of the specific 5-LOX pathway to overall airway inflammatory pathology, consequently reducing the clinical efficacy of both 5-LOX enzyme inhibitors as well as CysLTR1 receptor inhibitors (Kalayci et al., 2006). The CysLTR1 G300S variant confers a stronger potency to the potent bronchoconstrictor LTD4, and may confer low response to CysLTR1 receptor antagonists at conventional doses (Thompson et al., 2007; Duroudier et al., 2009)

Opioid receptors

Opioids exert their actions through their agonistic properties on three major classical GPCRs, namely the μ-receptor coded by the OPRM1 gene at 6q24-q25, the κ-receptor coded by OPRK1 at 8q11.2 and the δ-receptor coded by OPRD1 and located at 1p36.1-p34.3. These receptors are differentially expressed, exist as different subtypes, and mediate the various opioid effects such as analgesia, euphoria, dysphoria and dependence. A fourth receptor, orphanin FQ, encoded by the OPRL1 gene located at 20q13.33, is also known to be activated by opioid ligands.

The A118G, (rs1799971, allelic frequency=0.19) SNP in the OPRM1 gene has probably been the most studied. Molecular work suggests that the G allele results in a gain of function of the μ-receptor to the endogenous opioid β-endorphin, but a loss of function to exogenously administered opioids such as morphine. This has been corroborated with clinical evidence, where the G allele has been associated with a reduced morphine response in cancer and post-operative patients. However, in a meta-analysis of opioid pain studies, the authors could only detect weak associations with increased morphine dose requirements in homozygous carriers of the variant G allele. This suggests that clinical opioid response

may be the result of complex interactions between various pharmacogenetic variants. It could also be due to other common functional variants which have not been studied during meta-analysis and could therefore confound the outcome. (Mura et al., 2013; Crist and Berrettini, 2013). For example, *in vitro* studies suggest that another common variant, the exonic OPRM1 C17T, may be a relevant marker of low buprenorphine efficacy in opioid dependence management therapy (Bajada, 2010). Moreover, opioid response is known to also be influenced by other genetic polymorphisms, besides those affecting the receptors themselves. Such genes include those that code for metabolic enzymes such as CYP2D6, CYP3A4, CYP3A5, UGT2B7 and membrane transporters such as ABCB1.

4.1.2 Nuclear receptors

The nuclear receptor family, although much smaller than the GPCR group, comprises important targets for pharmacological management.

Glucocorticoid receptor

The human glucocorticoid receptor (hGR), a 94-kDa protein encoded by the NR3C1 gene located at 5q31-q32, has received significant pharmacogenetic attention, mainly due to the wide array of glucocorticoid agonists available in the therapeutic repertoire. Glucocorticoid responses are the fruit of complex multiple pathways, involving the activation or repression of multiple genes, and genotype-phenotype associations, are therefore often very difficult to establish. Moreover, although the human genome only contains one known glucocorticoid receptor gene, there are several receptor isoforms which are the result of polymorphisms, alternative splicing and alternative translational initiation. Furthermore, hGR isoforms may also be subject to a variety of posttranslational modifications, resulting in altered receptor function.

Within a sample of 240 individuals, Niu and co-workers identified 108 polymorphisms present in a range of exonic, intronic and untranslated regions of the NR3C1 gene. Subsequent functional analysis of a candidate subset of these variations identified some of these regions to be associated with higher hGRα protein expression (Phe(65)Val and Asp(687)Glu), some associated with decreased ligand binding affinity (Ala(229)Thr and Ile(292)Val) and others to be associated with decreased hGRα transcript expression (746T>C and haplotype 237delC / 238C>T / 240G>C). These functional polymorphisms were present in allelic frequencies in the range of 5.8% and 18.3% in different ethnic groups, and could contribute to reduced glucocorticoid sensitivity in a clinical setting (Niu et al., 2009).

The major issues associated with glucocorticoid therapy relate to the potential adverse effects (e.g. hypothalamic-pituitary-adrenal axis suppression, development of cushingoid features, hyperglycaemia, easy bruising, immunodeficiency) and to a patient subset who wish to refrain from this treatment. Both variables may not necessarily be directly associated to allelic variation within the NR3C1 gene, but may also be the result of downstream effectors which are responsive to hGR-induced pathways, or metabolic pathways which modify the pharmacokinetics of these drugs. For example, allelic variations in 8 genes (CNTNAP2, LEPR, CRHR1, NTAN1, SLC12A3, ALPL, BGLAP, APOB) have been associated with prednisolone-induced hypertension following a 28-day treatment remission induction of acute lymphoblastic leukaemia in children (Kamdem et al., 2008). Most of these genes are involved in the hypothalamic-pituitary axis pathway. Furthermore, as discussed earlier, gain of function polymorphisms in the ABCB1 gene, coding for the Pgp efflux transporter, have also been reported to reduce glucocorticoid efficacy by actively lowering intracellular glucocorticoid concentrations.

The main cellular hGR signalling activity is attributed to the ubiquitly expressed wild type isoform, hGRα. The β-isoform, a shortened splice variant of NR3C1, is also ubiquitly expressed, though at a lower level than hGRα. High expression levels of hGRβ are strongly associated with glucocorticoid resistance. The mechanism of this has been subject to many debates, especially since in contrast to hGRα, the β-isoform interacts poorly with heat shock proteins, does not bind ligands, and is transcriptionally inactive. The postulated mechanisms have included competition for hGRα binding sites (glucocorticoid responsive elements) on gene promoters, direct inactivation of the hGRα isoform by heterodimerization, and the inhibition of co-activating proteins which are necessary for hGRα activity. More recent evidence suggests that hGRβ competes with hGRα for binding to glucocorticoid receptor-interacting protein 1 (also called nuclear receptor coactivator 2 and coded for by the NCOA2 gene), thus generating an ineffective co-activator complex. Furthermore, a recent paper (Vazquz-Tello et al., 2013) has reported evidence showing that the cytokines IL-17 and IL-23, released by infiltrating T-cells in asthma, may contribute to hGRβ upregulation and consequently but only to a degree also contribute to glucocorticoid insensitivity.

The differential expression of the various hGR isoforms is driven by the selective use of at least 5 recognised hGR promoters (Russcher et al., 2007). The activity of these transcripts is further dependent on cell types and on promoter polymorphic variation. For example, the G allele of the BclI hGR promoter poly-

morphism is significantly associated with GC resistance and with the development of severe asthma (Pietras et al., 2011). The -22C>A polymorphism, located upstream of the hGR gene, causes a significantly lower transcriptional activity compared to the wild type C allele, as determined by promoter luciferase reporter assays in HepG2 and HEK293 cells, and is likely to be related to lower hGRα expression in the clinical setting.

The elucidation of clear hGR-associated pharmacogenetic genotype-phenotype relations remains a challenge. The complex molecular network involved in the outcomes of glucocorticoid receptor activation, suggests that the search for glucocorticoid pharmacogenetic variants should be spread over a large gene subset. Moreover, the effects of polymorphisms on the interrelated functions of these gene networks might be more important than the genetic variability present in the glucocorticoid-interacting receptor alone.

4.1.3 Enzyme pathways

The arachidonic acid cascade

Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids of cell membranes. It is released from phospholipid molecules through the actions of phospholipase A2 (PLA2) and is subsequently metabolized by a variety of pathways to products which are normally necessary for cell function and development, but this may cause pathologic manifestations if overproduced. The cycloxygenase (COX) pathway, mainly driven by the enzymes COX1 (coded for by PTGS1) and COX2 (coded for by PTGS2), in particular, is a target for a wide array of non-steroidal anti-inflammatory drugs (NSAIDs), which act to inhibit their activity. The PTGS1 gene contains two strongly linked SNPs (A842G and C50T) which when present in heterozygous patients, have been shown to significantly increase the susceptibility of COX1 for inhibition by aspirin. This carries the risk of increased aspirin toxicity at conventional doses, since many NSAID adverse effects are related to over suppression of COX activity. For example, NSAID-induced peptic ulceration is associated with over suppression of COX-mediated prostaglandin E2 (PGE2) production, and high anti-platelet effect is associated with excessive inhibition of COX-mediated thromboxane A2 (TXA2). On the other hand, several COX polymorphisms (PTGS1: C22T, C50T, A842G, G128A, C644A, C714A, C10427A, G1446A; PTGS2: G765C) have been reported to cause resistance to the pharmacological effects of aspirin (Xu et al., 2012). The arachidonic acid pathway is not the sole source of NSAID-related pharmacogenetic variability. For example, lumiracoxib, (a COX2-selective NSAID) hepatotoxicity has been strongly associated with the human leukocyte antigen haplotype HLA-DRB1*1501-HLA-DQB1*0602-HLA-DRB5*0101-HLA-DQA1*0102 (Singer et al., 2010).

The lipoxygenase pathway

The enzyme 5-lipoxygenase (5-LOX) metabolizes arachidonic acid to a family of leukotrienes, amongst which the most important are LTB4, LTC4 and LTD4. All of these exhibit bronchoconstrictory and mucosecretory properties within the respiratory tract, and have been shown to be important mediators of airway inflammatory diseases, such as asthma and allergic rhinitis. 5-lipoxygenase is coded for by the ALOX5 gene located at 10q11.2, and the enzyme is activated by the 5-lipoxygenase activating protein (ALOX5AP gene located at 13q12). Transcriptional regulation of ALOX5 is heavily dependent on a pentarepeat SP-1 binding motif repeat located in its promoter. Lower or higher numbers of ALOX5 promoter SP-1 repeats have been shown to significantly reduce ALOX5 gene expression. Indeed, asthma patients carrying a variant SP-1 repeat number show minimal or no improvement following treatment with zileuton, a 5-LOX inhibitor, due to the reduced expression of the drug target. The functional outcome of this polymorphism can be compounded further by concomitant polymorphic variation in other pathway-related genes, such as 5-lipoxygenase activating protein (coded for by ALOX5AP), leukotriene A4 hydrolase (coded for by LTA4H), leukotriene C4 synthase (coded for by LTC4S), and the leukotriene receptors CYSLTR1 and CYSLTR2. (In et al., 1999; Drazen et al., 1999; Telleria et al., 2008; Geiger et al., 2009; Duroudier et al., 2009; Tantisira and Drazen, 2009). Interestingly, the ALOX5 haplotype [-1708G]-[21C]-[270G]-[1728A] appears to contribute to aspirin sensitivity in asthma, and may potentially help to identify aspirin-sensitive from aspirin-tolerant asthmatic patients (Choi et al., 2004).

Due to the common arachidonate substrate of the COX and 5-LOX enzymatic pathways, events occurring within one pathway can also influence the other. For example, COX-inhibition by NSAIDS enables the unmetabolized arachidonic acid to shunt to the 5-LOX pathway, thus generating higher amounts of bronchoconstrictor leukotriene products. This has been the basis of the bronconstrictory adverse effect of NSAIDs often seen in aspirin-intolerant asthmatics; an effect which is exacerbated by the presence of genetic variants which influence the 5-LOX arm of arachidonate metabolism. The first reported such polymorphism was the leukotriene C4 syntase (LTC4S) promoter SNP (-444 A/C), which was associated with aspirin-induced asthma exacerbation, potentially due to LTC4S-mediated increased leukotriene production in carriers of this allele (Sanak et al., 1997). This association, which was derived from a small adult patient-based

study, has unfortunately not been replicated by other studies. In addition, luciferase-based promoter reporter experiments in HeLa cells and KU812F cells have not been able to identify any influence of these polymorphisms on promoter transcriptional activity. However, this SNP does seem to contribute to lower pulmonary function FEV1 readings in children (Sayers et al., 2003).

Tyrosine kinases

Tyrosine kinases constitute a family of enzymes that owe their importance to their tight involvement in proliferative cell signalling and cancer management. Kinase variants provide a robust way to enable the classification of patients suffering from several types of cancers, into specific therapeutic groups, based on pharmacogenetically-based predictive therapy outcome. Genotyping of tyrosine kinase variants has applications in predicting therapy efficacy as well as drug resistance. This may be exemplified by the characterisation of the BRC/ABL translocation in Chronic Myeloid Leukaemia (CML). The development of imatinib, a small molecule tyrosine kinase inhibitor, binding specifically to the ATP pocket of the BCR/ABL kinase domain, is intended for use in BCR/ABL positive leukaemia.

The characterization of the molecular mechanism of disease thus allows for the development of patient-targeted therapy. Targeted therapy has been implemented in the clinic and includes the use of the monoclonal antibodies rituximab and trastuzumab to target CD20 positive lymphomas and HER2 positive breast cancer cases respectively. Small molecule inhibitors, such as imatinib, were designed to specifically inhibit the tyrosine kinase fusion protein bcr/abl in CML. In addition, imatinib inhibits the receptor tyrosine kinase, cKIT and hence targets activation of cKIT mutants in Gastrointestinal Stromal Tumours (GIST). Table 2 lists current therapies used to target specific genetic aberrations.

Table 2: Examples of genetic variants that are used to enable optimum selection of specific oncology therapy. These biomarkers, which may be identified by appropriate genotyping, can predict whether the drug will show an adequate response or whether the patient will be resistant to its actions. The FDA advocates in favour of predictive genotyping for the intended use of these drugs for targeted therapy.

Drug	**Drug Target**	**Disease**	**Resistance to Therapy**	**Reference**
imatinib	BCR-ABL	Chronic Myeloid Leukaemia	BCR-ABL mutations	(Gorre et al., 2001; Zhang et al., 2009)
imatinib	cKit receptor	Gastrointestinal Stromal Tumours	KIT V654A	(Rubin et al., 2010; Chen et al., 2004)
trastuzumab pertuzumab	HER2 receptor	Breast Cancer	PIK3CA mutants	(Kataoka et al., 2010)
vemurafenib	BRAF V600E	Metastatic Melanoma	RAS mutations (activation of MAPK)	(Chapman et al., 2011)
erlotinib	EGFR L858R; Δexon 19	Non-Small-Cell Lung Carcinoma	EGFR (T790M)	(Miller et al., 2012; Kwak et al., 2010)
cetuximab	EGFR	Colorectal Cancer	KRAS mutations	(Liévre et al., 2006; Allegra et al., 2009)

Activating tyrosine kinase mutations are central to specific targeted therapy. Investigation of kinase deregulation within particular patient groups, has led to identification of mutant tyrosine kinases associated with disease progression and therapy modulation. Of interest is the identification of mutations in the receptor tyrosine kinase cKit, associated with high relapse risk in core binding factor leukaemias (Paschka et al., 2006). Mutations in exon 8 and 17 of the KIT gene significantly decrease the overall survival of acute myeloid leukaemia patients with the translocations inv(16) and t(8;21). The potential specific targeting of cKit using small molecule tyrosine kinase inhibitors necessitates the promotion of screening patients with core-binding factor AML for KIT mutations. This genetic profile of specific groups of patients gives prognostic and predictive information of clinical relevance (Cammenga et al., 2005).

In addition to high relapse rate, therapy outcome markers include mutations that predict resistance to therapy. In colorectal cancer (CRC) treatment, K-Ras mutations significantly predict resistance to anti-EGFR therapy. Several clinical studies have shown that the presence of a K-Ras mutation is a significant predictor of resistance to anti-EGFR therapy in colorectal cancer patients (Liévre et al., 2006). The identification of K-Ras mutants in CRC became part of the clinical practice protocols and clinical trials. Table 2 presents a list of mutations that are increasingly being used to pre-

dict therapy outcome. There are various mechanisms of resistance that account for primary and secondary (acquired) lack of therapy response. Point mutations in the kinase domain of the target molecule conferring steric hindrance account for the majority of primary resistance.

5 Analytical platforms for pharmacogenetics

For several years, genotyping technology has only been accessible through the expertise and hardware platforms of specialized laboratories. There is now a gradual movement to bring pharmacogenetic genotyping technology to the bedside, through portable automated equipment, which transfers most of the onus of obtaining good analytical quality data to the instrument, rather than the operator. An example of this is the patented FDA-approved Spartan RX CYP2C19 test, which analyses *2, *3, and *17 alleles, and is specifically relevant to CYP2C19-metabolized substrates, such as the antiplatelet drug, clopidogrel. The portable genotyping device uses a buccal swab as the analyte and delivers genotyping results within 60 minutes, with minimal operator intervention.

Larger scale genotyping assays remain within the domain of specialized centres. For example, the Affymetrix DMET+ microarray chip is capable of simultaneously analysing a panel of 1936 pharmacogenetically-relevant mutations from 225 different genes consisting of 50 CYP450 genes, 45 Phase II enzymes, 64 drug transporters and 66 transcriptional regulators and enzymes. The Affymetrix Amplichip, marketed by Roche, uses similar microarray technology applied on a smaller scale, to provide genotype data of a panel of CYP2D6 and CYP2C19 polymorphisms which are relevant to the metaboliser status for specific β-adrenergic receptor inhibitors, antidepressants, anti-psychotics, proton-pump inhibitors, anti-epileptics and opioids.

It is the challenge of scientists and technology to place more pharmacogenotyping tools in the hands of drug prescribers, to further enhance reaping the benefits of personalized medicine.

6 Conclusion

Over one hundred drugs currently approved by the FDA, require the inclusion of pharmacogenomic information on the drug label (FDA, 2013). This translates to approximately 25% of patients who make use of hospital outpatients services, being prescribed one or more drugs that have pharmacogenomic information on the labelling (Frueh et al., 2008). For these therapeutic groups, clinically relevant information obtained using pharmacogenetic biomarkers is a warranted approach to allow proper classification of patients into predictive therapeutic categories. Integrating this information into patient management protocols allows selection of personalized targeted treatment regimes, reduces interpatient drug response heterogeneity, saves unnecessary drug toxicity and decreases morbidity.

In order to accomplish this target, multiple approaches need to be conducted in parallel. Pharmacogenetic data must be validated through repeatability studies and cumulated into curated repositories. Indeed, such repositories have rapidly developed in the last 10 years (Patrinos and Brookes, 2005; Lagoumintzis et al., 2010). Besides the "Table of Pharmacogenomic Biomarkers in Drug Labeling" provided by the FDA (FDA, 2013), other noteworthy repositories are the PharmGKB Pharmacogenomics Knowledgebase, managed by the PharmGKB team at Stanford University, California, USA (Whirl-Carrilo et al., 2012) (Pharmacogenomics Knowledgebase (PharmGKB), 2013) and FINDbase, maintained by the GoldenHelix Institute of Biomedical Research, London, UK (Georgitsi et al., 2011) (FINDbase Genome Variation Allele Frequencies Worldwide, 2013).

Actions toward translational pharmacogenetics are needed (Squassina et al., 2010). Indeed, large scale initiatives to consolidate pharmacogenomic data and translation of these from bench to bedside are currently underway. The Pharmacogenomics for Everyone (PGENI) initiative aims to focus pharmacogenetic data into a country-specific knowledgebase in order to augment the relevance and applicability for individual patients (Pharmacogenomics for Everyone Initiative (PGENI), 2013). The Clinical Pharmacogenetics Implementation Consortium (CPIC), established in 2009, is a shared project between PharmGKB and the Pharmacogenomics Research Network. CPIC publishes peer-reviewed guidelines to pharmacogenetic-guided drug dosing, and simultaneously posts these recommendations to the online curated PharmGKB repository (Clinical Pharmacogenetics Implementation Consortium (CPIC), 2013).

Pharmacogenetic stratification of patients in clinical trials opens a new door to enable early identification of specific drug responses in a genetically defined patient subgroup. The inclusion of such stratification, from drug development Phase II clinical trials onwards, generates drug outcome data that may be otherwise genetically confounded and therefore not statistically identifiable. This is especially relevant for diseases such as asthma, for which therapies directed at new drug targets are currently under development (Portelli and Sayers, 2012). This approach has to be combined with the use of new large scale high throughput omics-based technologies, in order to identify novel functional genetic

loci, which either on their own or in combination, can be used as predictive biomarker panels.

Pharmaceutical and diagnostic companies are working together to formulate robust pharmacogenetic tests which could be used to predict the best drug at the right dose for a specific patient. This goal aims to achieve safer and more effective patient outcomes and in the long term, decrease pharmacoeconomic-related costs. Various issues including complex genotype-phenotype associations, variable penetrance, gene-gene interactions, and ethnic-dependent differences in allelic distributions, make this a very challenging task. Its success depends entirely on the concerted efforts of industry, academia and regulatory agencies, combined with the provision of relevant educational services for drug prescribers and associated healthcare providers.

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Review Article

The Optimism Bias: A cognitive neuroscience perspective

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Abstract. The optimism bias is a well-established psychological phenomenon. Its study has implications that are far reaching in fields as diverse as mental health and economic theory. With the emerging field of cognitive neuroscience and the advent of advanced neuroimaging techniques, it has been possible to investigate the neural basis of the optimism bias and to understand in which neurological conditions this natural bias fails. This review first defines the optimism bias, discusses its implications and reviews the literature that investigates its neural basis. Finally some potential pitfalls in experimental design are discussed.

Keywords Optimism bias - cognitive neuroscience - psychology - neural basis.

1 Introduction

Let us assume that John is a very poor student. He has not studied at all for his upcoming exam and has even sat a practice test which he failed. Despite all of this evidence, John believes that his chances of passing the upcoming exam are very high. Since his expectation is better than reality he is being unrealistically optimistic. John exhibits the optimism bias. This definition causes a problem for researchers who want to study the optimism bias. The experimenter cannot possibly have access to all of the variables that will affect John's exam result. Hence, it is virtually impossible for an experimenter to accurately quantify an individual's probability of experiencing a particular event (Weinstein, 1980).

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2 The Study of the Optimism Bias

One way for scientists to test the optimism bias in the laboratory is to ask an individual to predict his chances of experiencing an event and then following up to see whether the event transpired. The problem with this approach is that the outcome of an event does not always accurately represent the person's prior chances to attain that outcome; this is especially true when the outcome is a binary one. For example, we know that an individual has an infinitesimally small chance at winning the national lottery. If Peter predicts that he has a 75% chance of winning next week's lottery and then happens to win the lottery, this does not mean that Peter was actually pessimistic in his prediction. It simply means that he was very lucky, over and above being very optimistic.

While it is extremely difficult to tell whether an individual is being unrealistically optimistic, it is relatively easy to show that, as a group, people are unrealistically optimistic (Weinstein, 1980). If it can be shown that the majority of people in a group believe that they are superior to the majority of other people in that group, then it can be inferred that some of these people are unrealistically optimistic (Sharot et al., 2012; Weinstein, 1980). For example, (Svenson, 1981) showed that 88 percent of US drivers believe that they are safer drivers than the median driver. People were also shown to think that they were more likely than their colleagues to like their post-graduate job or to own their own home. At the same time participants thought that they were less likely than their colleagues to have a drinking problem or to attempt suicide (Weinstein, 1980). People also remain unrealistically optimistic about their own futures despite clear evidence that they should not be.

3 Psychological Mechanisms Underlying the Optimism Bias

Classical theories of learning suggest that if a person is given accurate information that contradicts their belief, then that person should subsequently update their expectations in a Bayesian manner (Pearce and Hall, 1980; Sharot, 2011). In a study set up by (Sharot, 2011), it was found that healthy individuals update their expectations in an asymmetrical way. If participants were given news that exceeded their expectations, they updated their future expectations according to the classical learning theory. However, if the news was worse than they expected they updated their future expectations slightly, but this did not reflect the extent of the news (cf. Eli and Rao 2011). An illustrative example given in the paper was that if a participant expected that his chances of getting cancer was 40% and was told that his actual likelihood was 30% then subsequently, the participant would probably decrease his expectations of cancer to about 31%. If, on the other hand he estimated his likelihood of getting cancer at 10% but was told that the true likelihood was 30%, he would subsequently not update his expectation to 30% but perhaps update it to about 14%. Evidence also shows that both younger and older individuals exhibit a greater asymmetry in belief updating. Hence, children and elderly individuals tend to have problems learning from bad news (Chowdhury et al., 2013; Moutsiana et al., 2013).

There is a group of individuals that do not show this bias. (Strunk et al., 2006) showed that dysphoric, or mildly depressed, individuals do not show any bias (optimistic or pessimistic). It is important to note that this does not necessarily mean that a dysphoric individual is always realistic, but that on average any biases they have cancel each other out. The authors also found that as a person becomes more depressed they are more likely to show a pessimistic bias.

As a result, experimenters have argued that healthy individuals have a tendency to be optimistically biased. This is in line with animal experiments that showed healthy, well treated animals to be optimistically biased whereas animals in poor environmental condition did not show the bias (Matheson et al., 2008). Traditionally, psychologists have maintained that a realistic outlook on life is the hallmark of mental health and wellbeing (Taylor and Brown, 1988). (Lazarus, 1983) started questioning this fundamental tenant of mental health. One could imagine a scenario were two cavemen, Peter and Max, are sitting in their cave and hear a rustle outside. This same sound could represent food, a little edible squirrel, or alternatively it could be a predator, the soft rustle of a lion's paws over leaves. In most situations it is a squirrel but occasionally it is a lion. Peter is optimistic and goes out; Max is pessimistic and stays in. Which one is better off? In this case Peter got eaten by the Lion, but if Max never leaves the cave he will starve to death. (McKay and Dennett, 2009) argue that although misbeliefs in general are maladaptive for an organism, positive false beliefs, such as the optimism bias, are generally adaptive. In fact, there is an increasing amount of studies showing that people who exhibit moderate levels of unrealistic optimism are better off than counterparts who have no bias, a pessimistic bias or an excessively optimistic bias (Friedman et al., 1995; Puri and Robinson, 2007). For example, optimistic individuals are more likely to comply with medical treatment and attend follow-up appointments (Friedman et al., 1995; Scheier et al., 1989). (Varki, 2009) takes an extreme point of view and proposed that optimism is not only useful but is indeed essential for human beings to function properly and survive. He states that with the ability to prospect comes the knowledge that death awaits each and every one of us. He proposes that without an unrealistically optimistic outlook on life humans would be overcome by great fear that would essentially render us extinct. Hence, the optimism bias is adaptive and likely to have been evolutionarily preserved for two crucial reasons. First, despite being inaccurate in their predictions of future events, optimistic individuals are more likely to be motivated to improve their wellbeing. For example, if an individual thinks they are less likely than the average person to contract disease X they may actively find ways to ensure that they do not, thus the prediction becomes a self-fulfilling prophecy. Second, they are less likely to be overwhelmed by an existentialist crisis that could lead to suicide. The increase in the optimism bias in elderly individuals may be a mechanism for elderly individuals to cope with the increasing health problems that arise in old age (Chowdhury et al., 2013).

Although moderate unrealistic optimism can indeed be adaptive, excessive optimism has, on the other hand, been shown to be maladaptive. Collective unrealistic optimism has been, in part, blamed for some of the greatest economic follies of our time such as the economic bubbles (and their inevitable crash) (Johnson and Fowler, 2011). While moderate optimists are generally selective risk takers and make relatively good economic decisions, extreme optimists tend to make decisions that are generally considered to be unsound (Puri and Robinson, 2007). In fact, the increase in optimism in older age may be a double edged sword since although the higher optimism allows the elderly to cope with increasing health problems, it may also lead them to make poor financial decisions (Chowdhury et al., 2013).

4 The Neural Basis of the Optimism Bias

If this bias is found in most healthy individuals and has been evolutionarily selected for, then it is reasonable to assume that there is a neurological network that underpins it. (Sharot et al., 2007) published a pioneering paper on the neural basis of the optimism bias. Using functional MRI (fMRI), Sharot and colleagues demonstrated that the rostral anterior cingulate (rACC) and the amygdala showed enhanced activation when participants imagined positive future events. The authors suggest that the amygdala could be involved in the mental construction of future events that have a high emotional valence. It was also suggested that the rACC is involved in monitoring the subjective importance of a future event and it may reflect the brain's regulatory mechanism to steer the individual to select an optimistic outlook. The rACC was shown to be strongly functionally connected to the amygdala, suggesting an intercommunicating neural network underlying the bias for positive predictions. The authors also pointed out that these are the same regions abnormal in people who suffer from a depressive illness. The pessimistic bias shown in depressed patients may be due to a disruption of the above neural network. Although researchers have investigated functional brain networks there has been no research to date on the structural networks that underlie the functional networks. With the advent of advanced diffusion MRI tractography techniques, there is an opportunity for further research in understanding the brain network that underlies the optimism bias.

In their 2011 study, Sharot and colleagues also provide data on the neural mechanisms involved in maintaining optimism, in spite of evidence that one should not be optimistic. The authors suggested that the right inferior frontal gyrus (IFG) may be involved in monitoring negative information. Interestingly the right IFG of participants who scored low on trait optimism was better at monitoring negative information than in those participants who scored high on trait optimism. Conversely, the left IFG, the cerebellum, the left and right medial frontal cortex (MFC) and superior frontal gyrus (SFG) were involved in monitoring positive information. These areas did not show any difference in participants who scored high or low on trait optimism. The authors were able to predict the amount that a person would update his or her beliefs by looking at the reduction in BOLD activity in the right IFG. It was suggested that optimistic individuals have a reduced ability to neurally code aversive information. The authors of the study also suggest that the effect may be modulated by the participant's motivation to have the best future possible. The results were consistent with results in other domains of neuroscience, where it was shown that these areas are associated with behavioural and reality monitoring (Brunamonti et al., 2014; Sugimori et al., 2014).

The next important step was to identify whether any of these regions are necessary for the optimism bias to be present. While fMRI is a correlational method, Transcranial Magnetic Stimulation (TMS) uses high field magnetic pulses to directly interfere with the electrical activity of a neural structure. If it can be shown that a disruption in one anatomical structure results in a change in the effect of the optimism bias, then there will be a strong claim that the neural structure is necessary in generating the neural process that produces the behavioural effect known as the optimism bias. Using TMS (Sharot et al., 2012) showed that a disruption of the left IFG improved the participant's ability to learn from bad news. Participants who had TMS stimulation on the left IFG updated their expectations even when they received bad news. TMS to the right IFG did not show this effect. It may seem contradictory that, while the fMRI study suggested that the right IFG was involved in monitoring negative information, it was TMS to the left IFG that showed a change in behaviour. This can be explained if the left IFG has an inhibitory role (Anderson et al., 2004; Aron et al., 2004). If TMS inhibits the proper functioning of the left IFG it may cause a disinhibition of the system, thus eliminating the bias. This paper has shown a proof of principal that the optimism bias can indeed be modulated by interfering with the body's hardware (or wetware).

Research has also shed light on the possible neurotransmitters that are involved in modulating the optimism bias. (Sharot et al., 2009) showed that participants who received L-DOPA expected more pleasure out of future events than participants who have not received the drug (Sharot et al., 2009). It was also shown in a later experiment that an increase in dopamine increases the optimism bias by impairing the participant's ability to learn from negative outcomes (Sharot et al., 2012). In fact, the optimism bias increased as a function of dopamine levels. This evidence is consistent with the results of (Frank et al., 2004) who showed that parkinsonian patients that were off dopamine enhancing medication can learn better from negative outcomes, while patients on their medication do better learning from positive outcomes. These studies provide a possible mechanistic explanation behind dopaminergic antidepressant drugs (Papakostas, 2006). In summary, the research of recent years has shown that the optimism bias is associated with frontal (IFG) and limbic (ACC and amygdala) brain networks. It suggests that the left IFG is important in the inhibition of updating expectations in response to bad news. Finally it has been shown that dopamine is an important modulatory neurotransmitter

in the system and that the availability of dopamine in the brain affects the extent of the optimism bias.

Although the scientific community has started to understand the brain basis of the optimism bias, further questions remain unanswered. For example, is there a particular sub region within the IFG that is more important for this effect than other areas? The cytoarchitecture of the IFG is not homogenous (it can be divided into Brodmann areas 44, 45 and 47). Furthermore, language research has shown a double dissociation within the left IFG, with the anterior region being involved in semantic processing while the posterior region is implicated in phonological processing (Buckner et al., 1995; Fiez, 1997; Gough et al., 2005). Presumably, the anterior and posterior aspects of the IFG are involved in different structural and functional networks. It would be interesting to see whether the optimism bias shows specificity to a particular location within the IFG. This line of research could improve our structural understanding of the networks involved in producing this bias. It will also be interesting to see whether patients who have structural damage (secondary to stroke) to particular areas show predictable deficits (or enhancements) in the optimism bias. Do people who have acquired aphasia due to a stroke in the left IFG show a reduced optimism bias? This information would be invaluable to practicing clinicians involved in the rehabilitations of these patients.

5 Criticisms and Caveats

Although the optimism bias has been studied extensively since the 1980s, there have been a few caveats raised in the literature that cast doubt on some experimental designs. (Harris and Hahn, 2011) argue that three types of statistical artefacts may make them seem to be unrealistically optimistic.

First, the authors show experiments that use discrete attenuated scales rather than continuous scales that can cause an appearance of optimism when there in fact is none. This is because attenuated scales, for example a scale from 1 to 5, does not allow for subtle differences to be reported by the participant. Hence, even if participants are realistic in their true estimates, an attenuated scale can make them appear unrealistically optimistic.

Second, due to minority undersampling, if an event is rare enough, the likelihood of finding people who know that they have a higher probability of experiencing that event is rare, thus, since the minority is underrepresented, our data will not show the true picture.

Finally, base rate regression is when people tend to overestimate the risk that an event will occur to the average person. Hence, an accurate prediction by the participant of an event happening to them may be interpreted as optimistic due to an overestimation of how probable that event is for other people.

These limitations are mainly present in experiments where groups of participants compare their risk to another group of individuals. For example, when on average people think that they are better drivers than the average driver. (Shepperd et al., 2013) argues that while these statistical limitations look worrisome, they should not cause undue concern. First, experiments using non attenuated scale have still showed evidence, albeit less dramatic, of the optimism bias. Second, minority undersampling is only a problem when investigating rare events and many experiments investigate relatively common events such as divorce, buying a nice house or getting a good job (Weinstein, 1980). However, even for these events, if many groups of people are examined, for example in a meta-analysis, minority undersampling disappears as a problem since the group will then be large enough to adequately represent rare events. Finally, experiments still showed evidence of the optimism bias when the base rate of an event was given, hence controlling for base rate regression.

Rather than disproving the optimism bias theory, the (Harris and Hahn, 2011) paper highlights potential pitfalls that any experimenter should keep in mind prior to designing their experiments.

6 Conclusion

In conclusion, the optimism bias is a well-established psychological phenomenon that, despite criticism, has been replicated in many experiments. While it is generally an adaptive phenomenon, it can have disastrous consequences (such as an economic collapse). Research has moved away from a description of the phenomenon onto trying to understand the underlying psychological and neural mechanisms that underpin it. This has led to investigation of ways of modulating this phenomenon. The translational potential of this research has already been recognised by some. Governments and institutions are, in certain cases, modifying plans to accommodate for it. For example, the budget for the 2012 London Olympics was modified to accommodate the optimism bias (House of Commons Committee of Public Accounts, 2008, p. 8; Sharot, 2012). This research has the potential to be translated from bench to bedside. It is providing a psychological and neuroscientific grounding for the treatment of depression. In a future where humans have the ability to remove or enhance an optimistic bias through pharmacological means or by TMS, the difficulty may be in recognising when the optimism bias is adaptive and when it is detrimental. Getting it wrong may, in itself, have disastrous consequences.

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Research Article

Skin Grafts: Local quest for viable alternatives to autologous grafts using silk and acellular dermal matrices

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Abstract. The gold standard with regards to skin transplantation is the use of the patient's own skin obtained from a healthy donor site. Such grafts can be either full thickness skin or more commonly nowadays, split thickness skin. Various materials, having either natural and or synthetic origins, have been used in the engineering of skin substitutes to-date and these grafts are then confronted against autologous skin grafts. If proven to be successful, such matrices could be utilised in clinical applications such as in the treatment of burn wounds and in cases of skin ulcers amongst others.

In this study the primary cells used, keratinocytes and fibroblast, were obtained from donor skin and cultured. Scaffolds of xenogenic (raw silk) as well as of allogenic (acellular dermal matrices) origins were obtained via low-cost methods and seeded using the fibroblasts and keratinocytes so as to determine which gave the closest mimic to skin grafts.

Out of the matrices assessed, the raw silk matrix allowed the best colonisation with skin cells in our hands. The ADM matrice also showed some cell colonisation, but will need further experimentation.

Keywords Skin graft, silk and acellular dermal matrix (ADM).

1 Introduction

The skin is the largest single organ of the human body. It is composed of the epidermis, which is an epithelial layer of ectodermal origin, and the dermis, a layer of connective tissue, originated from the mesoderm. The epidermis consists of a stratified squamous keratinized epithelium, as well as less abundant cells types namely; melanocytes, Langerhans cells, and Merkel's cells. The keratinising epidermal cells are called keratinocytes. Amongst many of its complex roles, one of its major functions is to protect the body against environmental influences. Acute or chronic loss of this barrier necessitates the mechanisms of tissue repair for any organism to survive. The most important step of such a mechanism in the re-epithelialization of the wounded surface is the primary destination of skin wound healing (Slavin, 1996).

It was Revendin who introduced skin grafts for the first time in 1871. Transplantation of the patient's own skin from a healthy donor site, either as a full thickness or as a split thickness skin graft, is commonly practised nowadays and is regarded as the surgical "gold standard" to cover skin wounds. This is the reason every tissue engineered skin substitute is always confronted against the performance of autologous skin grafts.

Knowing that extensive wounds necessitate a barrier protection to prevent both infection and desiccation, as well as the need of cell guidance by dermal elements to maximize healing, has led to the evolution of both biologic and synthetic dressings and skin substitutes (Horch et al., 2001). In cases of substantial burns, the great extent of wound surfaces and considerable loss of skin necessitated the invention of various types of temporary or permanent skin substitutes. The clinical utility of cultured skin substitutes for wound closure has reduced the amount of donor skin required by at least 10 times when compared with conventional skin grafts, and has also reduced the number of surgeries required to harvest donor skin, while at the same time decreasing the time of recovery of those patients with severe burn injuries.

Theoretically, skin substitutes can be permanent or

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temporary; epidermal, dermal or composite; and biologic or alloplastic (synthetic). Biologic components are further subdivided into autogenous, allogenic, or xenogenic. Logically, research efforts of different groups are centred on several possible combinations of these traits, whereas practically, most designs rely on a permanent or temporary engraftment of the material. Researchers in this field have defined the common techniques according to their principal biological action in the patient (Horch et al., 2005):

- Temporary – in this case the material is designed to be placed on a fresh wound (partial thickness) and left until healed.
- Semi-permanent – here the material remains attached to the excised wound, and eventually replaced by autogeneous skin grafts.
- Permanent – in this type there is an incorporation of an epidermal analogue, dermal analogue, or both as a permanent replacement.

The increasing emphasis on rehabilitation and "quality of skin cover" has further valued this field. A skin substitute which has the properties of a dermis is the marker for gauging a permanent substitute. Allogenic or alloplastic skin substitute coverage as a temporary solution is necessary until definitive cover can be achieved (Gallico, 1990). Allogenic skin grafts may be completely integrated into the healing wound initially and bridge the critical time gap in the early phase of burn treatment, but over time they will irrevocably undergo immunogenic rejection (Zhao, 1992). In theory, the application of in vitro cultivated autologous skin substitutes is able to overcome this specific deficit of modern burn treatment and reconstructive surgery.

Numerous studies have been conducted to date with the aim of solving important hurdles in skin transplantation. Such a problem is posed by the need for large amounts of skin in cases of burns and chronic skin ulcers, with scarce donor sites. In certain trials, cells were cultured in vitro, where the use of both autologous (Terskikh and Vasiliev, 1998) and non-autologous (Bolivar-Flores and Kuri-Harcuch, 1999) cells were assayed. Also, the use of dermal substitutes or their equivalents containing bio-degradable bovine collagen, in association with autologous cells cultured in vitro, has been studied by Jansson and colleagues in 2001.

The matrices looked at in this study, were composed of the following;

1.1 Protein Matrices

In general, around 60 percent of the polypeptide chain exists as two regular secondary structures, namely a helices and the β-sheets. The remainder of the molecule is in random turns and coils. Thus these helices and sheets are the major internal supportive elements of the protein itself.

Proteins having structural roles in cells, and thus having to span over a large distance, generally have an elongated three dimensional structure and are commonly referred to as fibrous proteins.

1.1.1 Silk

Silk derived from the silkworm, Bombyx mori, has been utilised as a biomedical material for applications such as sutures for many decades. According to (Altman et al., 2003), silk has distinctive mechanical characteristics that may be utilised in the field of clinical repair options with many possible applications. Although bioincompatibility of these fibres has been the major draw back in their utilisation; it has been demonstrated that this was most likely due to some residual proteins which may have contaminated the silk, not to the nature of the fibres themselves.

Many structural proteins are composed of multiple layers of pleated sheets that provide toughness. Silk fibres consist mostly of stacks of antiparallel β-sheets. The fibres are flexible because these β-sheets can slip over one another. Also, their resistance to breakage is derived from the fact that the silk's peptide backbone is aligned with the fibre's axis. In fact, core silk fibroin has been shown to exhibit biocompatibility both in vivo and in vitro comparable with that of other widely used biomaterials such as collagen and polylactic acid (Altman et al., 2003). Also, this study underlines the fact that silk fibres posses numerous different side chains to which growth factors, as well as adhesion factors, could be added. Another advantage of silk fibres is that these may be genetically tailored, making silk even more viable for biomedical utilisation which is in fact what is presently ongoing.

The use of spider silk has also been intensively assessed by (Yager, 1997), amongst many others, in order to determine its structure and physical as well as chemical properties so that such silk could also be used as an application in the future as a matrix for tissue engineering.

Genetic engineering techniques have been utilised in the synthesis of recombinant spider silk fibroin-mimetic polymers that have been proven to possess excellent mechanical properties (Guerette et al., 1996), as well as an improved cell adhesion capacity, as shown by (Bini et al., 2006).

Applications of silk include being scaffolds for tissue engineering, especially after the recent results obtained in trying to produce both ligament and bone formation in vitro. (Altman et al., 2003) thus conclude that such studies support the great potential of silk as a biomaterial for future clinical applications. Other studies have underlined the fact that silk is very versatile with respect to biocompatabilty, biodegradation, controllable

degradation rates (Rice et al., 2005; Urist, 1965) and the potential of being turned into a number of different formats including gels, porous structures and fibres (Preda et al., 2013).

1.2 Acellular Dermal Matrix (ADM)

There have been many attempts to produce a dermal substitute. The most successful of these substitutes seem to be ones derived from either full or split thickness skin treated to remove all epidermal and dermal cellular components and appendages such as keratinocytes, fibroblasts, hair, sweat glands and smooth muscle (Walter et al., 1998).

This substitute should exhibit three very important properties namely; low antigenicity, the ability to vascularise rapidly and to be stable as a dermal template.

2 Methods used

2.1 Isolation of Fibroblasts and Keratinocytes

The skin (full thickness skin) was collected through informed consent of the patients during surgeries such as circumcisions, tummy tucks and facelifts. The skin was then cleaned and collected into 50ml tubes that had been filled prior to this with RPMI 1640 (10% FBS, 100µg/ml of streptomycin, 100U/ml of penicillin, 2.5µg/ml amphoteracin B) and stored at 4°C. These samples were processed within 36 hours under clean conditions. The method used was adopted from an established protocol by (Jones et al., 1996) and separation of epidermis and dermis was achieved overnight by dispase digestion at 4°C. The epidermis was cultured in Keratinocyte Growth Medium (KGM) (100µg/ml of streptomycin, 100U/ml of penicillin, 2.5µg/ml amphoteracin B) to obtain keratinocytes. The dermis was cultured in DMEM F12 (10%FBS, 100µg/ml of streptomycin, 100U/ml of penicillin) to obtain fibroblasts.

2.2 Silk

The silk matrices were made from layers of raw silk peeled off the cocoon of the mulberry moth Bombyx mori (with the aid of a scalpel blade no.24). These layers were cut into small pieces (5mm by 5mm), then wrapped in foil and autoclaved.

2.3 Acellular Dermal Matrices

First the epidermal layer of formalin-fixed cadaveric skin was scraped off using a scalpel. A modified version of the method described by (Walter et al., 1998) was then followed. Briefly, the skin was incubated overnight in 1M NaCl at 37°C with continuous agitation. Next the skin was placed in 0.5% sodium dodeycl sulphate (SDS) solution for 1 hour at room temperature, again with continuous shaking. Both solutions used contained 0.02% sodium azide so as to prevent microbiological growth. The acellular dermal matrices were then extensively washed using sterile PBS and were then transferred using sterile forceps into cryovials. Then, 1ml of sterile PBS was added and the sample was frozen at −20°C.

2.4 Seeding of the Matrices

The matrices were seeded, under sterile conditions, using a fibroblast suspension of around 1.5×10^6 cells/ml. Two weeks following the seeding of the silk matrix with fibroblast, the matrices were seeded with keratinocytes. In the case of the ADM, seeding with keratinocytes was undergone after four weeks. At this stage, a suspension of keratinocytes at a concentration of 1x106 cells/ml was used. The matrices were kept under microscopic observation for the following weeks.

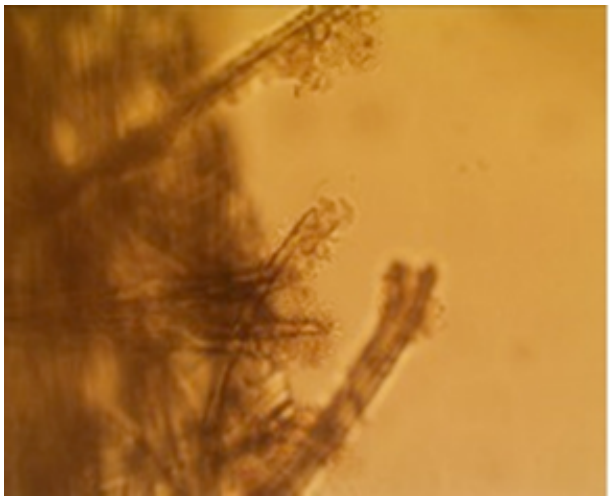

Figure 1: Cells attached all round silk fibres. (Magnification ×200).

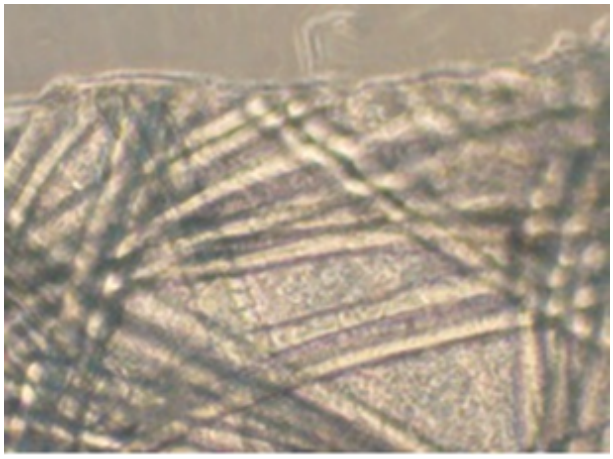

Figure 2: Spaces between silk fibres are bridged with cells. (Magnification ×400).

3 Results

3.1 Silk Matrix

Cells were seen to attach very rapidly into the silk fibres (Figure 1). At week six, that is, 30 days after the seeding of keratinocytes, cells were seen to spread out onto the silk fibres. Extended bridging between a relatively wide area of silk fibre was also observed (Figure 2).

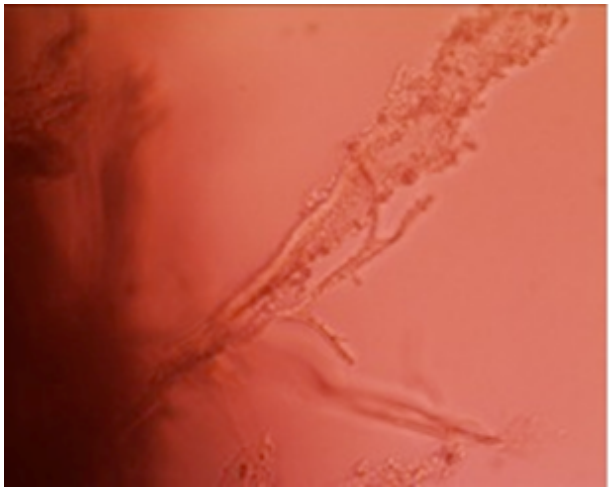

Figure 3: Cells attached onto the ADM's collagen strands. (Magnification $\times 400$).

3.2 Acellular Dermal Matrix

Cells were seen to grow compactly and uniformly onto collagen strands that spread out from the matrix's margins (Figure 3).

Next, the ADMs were stained with a Haematoxylin and Eosin (H&E) stain so as to investigate to what extent the fibroblasts and keratinocytes seeded attached onto the outer layer of the ADMs and whether the cells migrated deep into the inner layers of this collagen matrix.

4 Discussion

4.1 The Silk Matrix

The cells attached to the silk fibres relatively quickly when compared to the acellular dermal matrix. Also, over time the clusters of cells were seen to grow along the silk fibrils with frequent bridging between two or more of these structures. The cells were in fact seen to bridge even between not-so-close fibrils, over a period of four weeks, cells were seen to bridge over a relatively large span between multiple silk fibres, which was not seen in hair matrix cultures. Thus, silk seems to be relatively better in acting as a matrix onto which large films of cells form.

4.2 Formalin fixed Acellular Dermal Matrix

Unlike in the case of (Walter et al., 1998), who used fresh skin (stored at $-20°$C and thawed before the decellularisation process), formalin-fixed skin was used in this study since this was freely available in the department. Another study conducted by (Gibbs et al., 2006) prepared ADMs from glycerol-preserved door skin, thus formalin preserved ADMs might have been viable to achieve.

Our experience showed that both the decellularisation process, as well as the successive cell-seeding, were markedly less efficient with formalin-fixed skin and that this resource may need some treatment to reverse the effects of formalin (similar to the microwave antigen retrieval process for immunocytochemistry) to be a suitable source. Non-fixed skin (possibly derived from cosmetic procedures) is obviously a resource which should be investigated further.

With regard to cost effectiveness, although the acellular dermal matrices were cost-free to obtain, the reagents needed to process the matrices were both costly as well as relatively hazardous. While in the case of the silk, the cocoons were obtained at a very low cost and processing was practically cost-free. These facts helped make silk an ideal candidate for studies involving its utilisation as a matrix onto which skin grafts are moulded. Another point in this matrix's favour is the fact that core silk fibroin has been shown to exhibit biocompatibility both in vivo and in vitro, comparable with that of other widely used biomaterials such as collagen and polylactic acid (Altman et al., 2003). Combination matrices made from processed skin dermis, together with a silk framework or other related structure, may be future avenues to explore.

5 Conclusions

In the case of the acellular dermal matrix, being derived from formalin-fixed cadaveric skin, the formalin may have altered the protein surfaces rendering the adhesion of cells more difficult with respect to other matrices investigated.

The silk matrix seemed to be a good candidate for the development of skin structures, although studies have outlined the fact that raw silkworm silk can be immunogenic due to the protein sericin and that a degumming process should be carried out first (Kearns et al., 2008). Unfortunately, this in turn distorts the structure of the silk fibrons themselves and effects the mechanical properties (Langer and Vacanti, 1993), while sericin coated materials have been shown to aid cell adhesion and growth of human fibroblasts (Vacanti, 2001).

Acknowledgments

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Review Article

The Structure of Protein Molecules: In Celebration of the International Year of Crystallography, 2014

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Abstract. Many people, including laymen, are aware of the double helical nature of the DNA molecule. A few may actually realise that it was the technique of X-ray crystallography that was the key to solving this structure. Even fewer will understand the uses and applications of crystallography to the most diverse of biological materials; proteins. In this review we discuss the application of a number of methodologies required to progress from a cloned gene to protein expression and purification, crystallisation conditions and eventually to X-ray structure determination. We provide our own experience in the field as examples of the procedures required. Protein crystallographers worldwide are contributing to our understanding of how enzymes work, how our immune system defends us against viruses and are using structural information to design novel pharmaceutical reagents.

Keywords Protein Structure - Expression - Purification - X-ray crystallography.

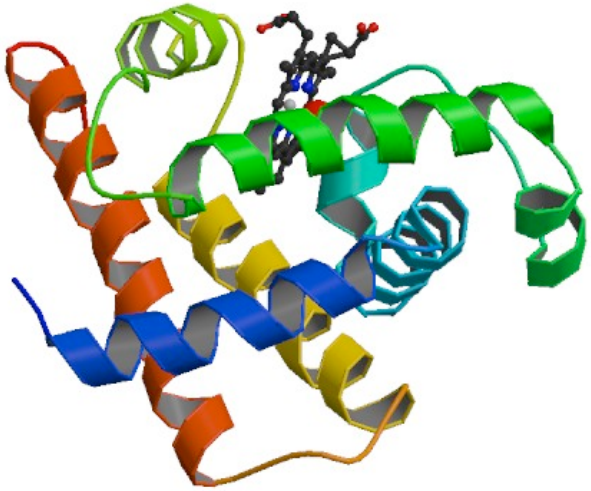

Figure 1: The first of many: the structure of the oxygen carrier protein Myoglobin from Sperm whale. Originally solved by Perutz, this is the structure from RCSB entry 1MBN, Watson, 1969. It clearly shows the position of the haem cofactor (ball and stick figure) and therefore where an oxygen molecule will bind.

1 Introduction

It is time for crystallography to step out of the shadows. Both to pay tribute to the contribution crystallographers have made to science and medicine, and to encourage young scientists in the field, UNESCO has declared 2014 as the International Year of Crystallography. Appropriately this celebration coincides with the centenary of the discovery of X-ray crystallography. The importance of X-ray crystallography to the advancement of science is apparent by the fact that since the first crystal was analysed by the Bragg father and son duo over 100 years ago at the University of Leeds, UK, 28 Nobel prizes, including the 2013 prize in Chemistry, have been awarded to X-ray crystallographers. Even in the early days of X-ray crystallography, interest was focused on biological molecules. The way in which structure helps to explain function was famously exemplified with the molecular model of DNA by Watson and Crick (Watson and Crick, 1953). Kendrew and Perutz were the first to apply the technology to proteins, namely myoglobin (Kendrew, 1962)(Figure 1; 1MBN, (Watson, 1969)) and haemoglobin, overcoming daunting obstacles to arrive at a three dimensional representation of these large molecules. Since then the field has exploded exponentially and now the repository of biological molecular structures, the RCSB (Research Collaboratory for Structural Biology, www.RCSB.org) which includes the protein data bank (PDB)(Berman et al., 2000) is fast

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approaching one hundred thousand entries. The importance of proteins in biological systems underlies the need to understand more about them, and protein structure determination should not be underestimated.

2 Protein Expression

Protein purification is an obvious primary requirement before crystallisation trials begin. Taken together, these are the two main bottlenecks frequently encountered in the process of determining the structure of a protein.

Isolation of protein from tissues may be cumbersome and laborious, requiring large quantities of source material and prone to high losses of protein. Although there may be good reasons for isolating proteins from their source organism (discovery of natural post-translational modifications, for example), the methodology of choice today is molecular cloning of the gene.

When information regarding the nucleotide sequence of the gene is available, proteins may be produced by recombinant DNA technology or by total gene synthesis. Expression of eukaryotic proteins starting with total messenger RNA entails the cloning and expression of the appropriate cDNA. This involves reverse transcription of messenger RNA, PCR amplification of the cDNA fragment of interest and cloning into an appropriate molecular vector. Even when genetic information of the gene of interest is absent, all is not lost, however. Short stretches of amino acid homology at the ends of a protein may be utilized to design degenerate primers for PCR experiments. Indeed this is how we cloned the gene for SOD-3 from *C. elegans*, and subsequently the corresponding cDNA for expression studies (Hunter et al., 1997a).

There are various expression platforms suitable for different proteins. With a large selection of expression vectors designed for specific expression protocols and purification systems now available, it should be possible to find a system that works for almost any protein. It should be stressed however that the best system is often found by trial and testing. Certain eukaryotic proteins require post-translational modifications for their biological activity and may have to be expressed in eukaryotic hosts capable of performing them. The yeast *Pichia pastoris* is such an expression platform that allows human-like glycosylation.

Bacterial expression systems produce large quantities of proteins and are easy to culture and remain the system of choice for many protein scientists. Over recent years the technology of expressing mammalian proteins in bacteria has advanced greatly permitting the production of a variety of proteins. Toxic and membrane proteins, inclusion body formation and limitations of codon usage are all problems which are now largely solved (Graslund et al., 2008).

3 Protein Purification

As each protein is unique, different purification procedures have to be designed and tested. Even similar proteins or mutant proteins differing by one amino acid residue may require different strategies in order to obtain protein of sufficient purity for later processing. The greatest challenge in purification is the elimination of background proteins whilst obtaining a high yield of soluble, pure target protein. Traditional protein purification procedures are often successful, and include salting out (ammonium sulfate precipitation), ion exchange chromatography and gel filtration. A combination of these procedures is usually required (Figure 2, (Vella et al., 2014)). When the protein is naturally abundant purification can be relatively easy, hence the use of Sperm whale as the source of myoglobin by Perutz.

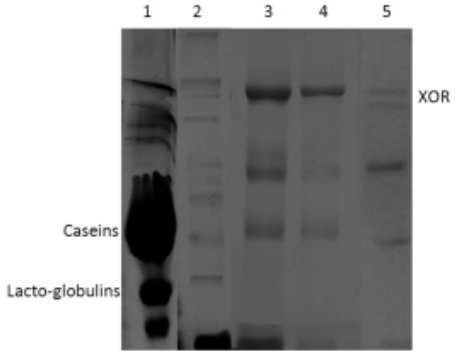

Figure 2: 15% SDS-PAGE illustrating the purification of xanthine oxidoreductase (XOR) from bovine milk. Lane 1 are proteins in fresh unpasteurized bovine milk, crude sample. Lane 2 is a protein sample following addition of 20% ammonium sulfate. Lanes 3 and 4 is a protein sample after chromatography on a heparin column. Lane 5 is a Protein sample after gel filtration chromatography. Purified XOR is shown as four bands on SDS-PAGE.

To expedite purification, affinity chromatography can be extremely effective, replacing a number of techniques with a single step and a plethora of affinity tags have been produced to utilize this powerful technique for almost any protein of choice. These tags are invariably incorporated into the target protein by genetic engineering, resulting in a chimeric fusion product. Removal of the tag after purification is also often an available option and again a number of protease restriction sites have been added to expression vector systems.

Commonly used tags include glutathione-S-transferase (GST) (Figure 3) (Hunter et al., 1997b), maltose-binding protein (MBP) or hexahistidine tags (Figure 4) (Hunter and Hunter, 2013) incorporated onto the N or C terminus of the protein being purified. Columns with immobilised glutathione, maltose or metals such as nickel are used respectively for pu-

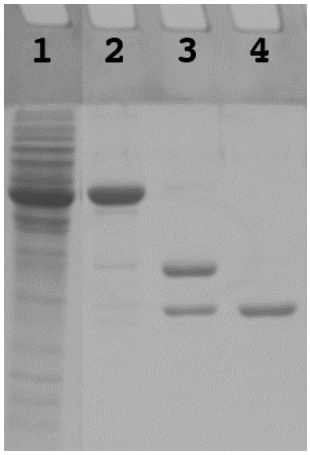

Figure 3: Expression and purification of a GST-SOD fusion protein. Lane 1 is the cell lysate, lane 2 is GST-SOD purified by GSH–sepharose chromatography, lane 3 is thrombin-cleaved GST-SOD showing both GST (upper band) and released SOD, lane 4 rechromatographed sample to remove the GST, leaving pure SOD as a single band.

rification of these fusions. Specific protease cleavage sites, including thrombin (Figure 3) and Factor Xa (Figure 4), may be engineered between the tag and the recombinant protein. This enables the cleavage of the tag away from the protein under study. A second affinity column removes the released tag (Figure 4) and many restriction proteases can be similarly removed to leave highly pure native proteins (Hunter and Hunter, 1998; Hunter et al., 2002). One disadvantage is that many vectors with engineered fusion tags will leave extra amino acids at the end of the protein, and to this end we developed a hexahistidine tag vector which leaves only authentic protein sequence after cleavage of the product (Hunter and Hunter, 2013).

4 Protein Characterisation

Once sufficient amounts of protein have been purified, which may be in the range of 20 to 50 mg, characterisation is carried out to confirm the identity of the isolated protein and to determine its biochemical properties to decipher the mechanism of function or catalysis. Absolute identity of the protein is confirmed by immunoblot with a specific antibody for the protein. Mass Spectrometry such as MALDI-TOF-TOF gives vital molecular weight information. This may confirm the occurrence of proteolysis or post-translational modifications. Purity is often assessed by SDS-PAGE and protein concentration is recorded using the BCA (Smith et al., 1985) or the Bradford method (Bradford, 1976), standard curves being made using BSA. A more accurate method of determining the concentration of pure protein is measuring the absorbance at 280nm and then calculating the concentration using the extinction coefficient by the Beer-Lambert law. Biological activity of the protein after purification is of paramount importance and often used to monitor the progress of purification procedures. Precisely what is measured will depend on the protein. With respect to metalloenzymes such as SOD it is necessary to measure cofactor metal content in order to calculate the specific enzyme activity. ICP-MS, MP-AES and AAS-GF are methods sensitive enough to detect such low levels of metals. Various spectrophotometric assays exist for the measurement of enzyme activity or native PAGE followed by zymography may be employed (Figure 5). Circular dichroism spectroscopy can provide information of any structural changes that may have occurred during purification, which could be detrimental to the activity of the protein. Gel filtration may be used to determine the molecular weight of the native protein which when combined with mass spectrometry or SDS-PAGE data can be used to calculate quaternary structure.

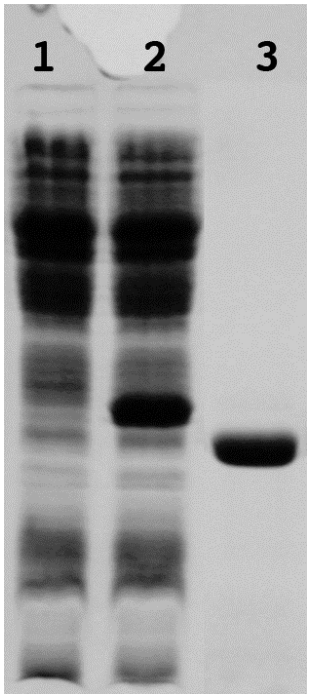

Figure 4: Expression and purification of a Hexahistidine-tagged SOD protein. Lane 1 is an E.coli cell lysate without the expression plasmid, Lane 2 is a cell lysate showing overexpression of the H6-SOD protein, Lane 3 shows the pure SOD band after nickel chelation affinity chromatography and cleavage by Factor Xa to remove the tag.

5 Crystallisation

The ultimate analysis for any biological molecule including proteins is the determination of its three-dimensional structure. X-ray Crystallography has been described as the technology that marries art with science. The objective is to produce crystals that are composed of regular, repeated arrangements of, in this case, a protein molecule. It should not

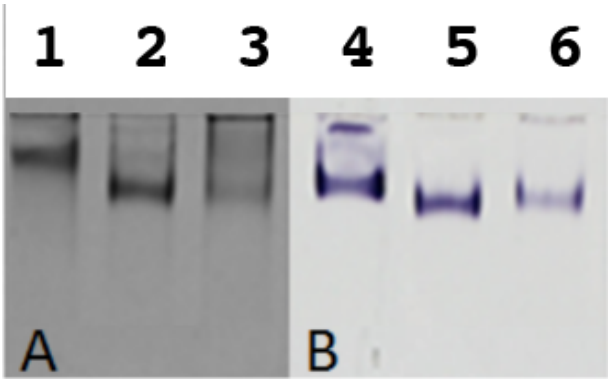

Figure 5: 8% Native PAGE followed by zymography. **A**: Native PAGE showing bovine, caprine and ovine XOR band (lanes 1 to 3 respectively) stained with Coomassie Brilliant Blue. **B**: Native PAGE after 2 hours of incubation in active solution showing bovine, caprine and ovine XOR band (lanes 4 to 6 respectively). Active XOR present on the gel catalyzes the conversion of xanthine to uric acid, producing hydrogen peroxide and superoxide anions. The latter reduce NBT to form a purple coloured insoluble formazan product

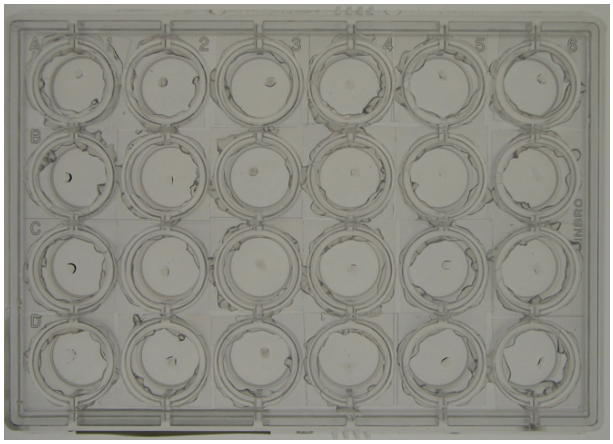

Figure 7: The hanging drop method of crystallisation. A drop containing reservoir solution and protein (1:1) is placed on an upside down coverslip over a well containing reservoir solution (precipitant, buffer and additives) and sealed. Vapour diffusion helps to produce crystals. Here a 24 well plate is used to test 24 different conditions for the same protein.

be forgotten that protein crystals are unnatural states for any protein, which helps to explain the difficulty in predicting the requirements to produce them. A 0.5mm cuboid crystal may contain as many as 10^{15} molecules of protein (Figure 6). The major ingredients that encourage protein crystal formation are a buffer and a precipitant. Initially the protein is diluted with this mixture and allowed to slowly equilibrate by vapour diffusion in a sealed chamber. This is most commonly achieved by the hanging drop method (Figure 7). Proteins may be co-crystallised with other molecules such as inhibitors, agonists, cofactors, nucleic acids and even other proteins. It may take months if not years to grow the perfect crystal.

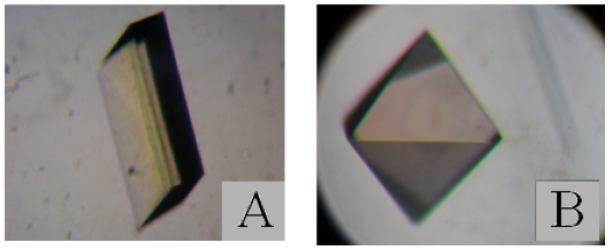

Figure 6: Crystals of superoxide dismutase. Crystal structures of similar proteins can produce surprisingly different crystals. *E.coli* FeSOD (A) and *C.elegans* MnSOD (B).

There are many variables involved in the crystallisation process and parameters that must be tested include protein concentration, temperature of the environment, humidity, pH, type and concentration of both precipitant and buffer and the inclusion of additives (almost any chemical compounds in existence). Consequently endless permutations may be possible and must be tested. For this reason hundreds of screening trials are carried out to identify the stringent optimal conditions that will reproducibly form stable and diffraction-quality crystals. Maybe out of sheer frustration, there are those who claim that other factors such as music and even supernatural phenomena affect the growth of that ever-elusive perfect crystal. The goal however is for a single crystal to form under reproducible conditions, which is large enough to diffract an X-ray beam effectively as the greater the diffraction angle, the higher the resolution of the final structure.

6 X-ray Diffraction

The X-ray diffraction pattern is essentially a series of spots of different intensities in different positions on a two dimensional detector (Figure 8A). A series of diffraction patterns has to be produced with the crystal mounted in the X-ray beam rotated by some small amount between data collection. Powerful computer programs utilizing Fourier transformation algorithms are used to convert this data into an electron density map (Figure 8B). The latter is effectively a three dimensional map of the position of all the electrons in the molecules.

More computing is required using the known sequence of amino acids in the protein to effectively fit the heavy atoms of the string of amino acids into the determined electron density (Figure 8C). This process, known as refinement, includes a reiterative technique that reduces errors to a minimum and produces what is accessible to all from the databank, the coordinates for the heavy atoms that make up the protein. A typical small protein like superoxide dismutase (MnSOD3) which has 194 amino acids produced 49,346 reflections and yielded the coordinates for 3569 heavy atoms including waters (pdb 3DC5) (Trinh et al., 2008). The later molecules are an intrinsic part of any protein, often determining how in-

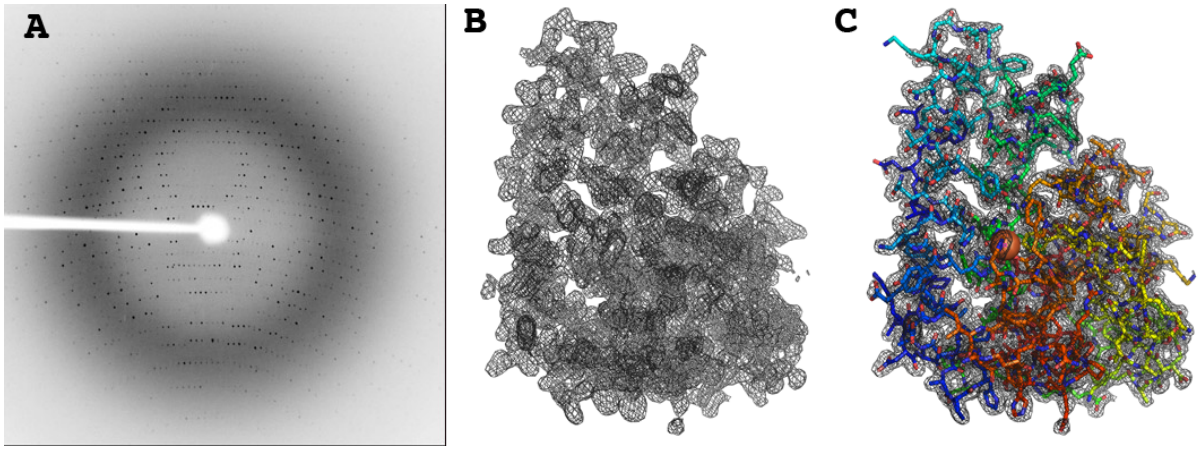

Figure 8: Generating a protein structure. The process begins with (A) the diffraction pattern produced by a protein crystal of FeSOD. (B) the electron density map (EDM) generated by Fourier transform of (A), and (C) the fitted amino acid sequence with the EDM.

teractions between the protein and substrate occur as well as protein:protein interactions in the quaternary structure. This is not quite the end of the process however as the structure returned by the above processes is the asymetric unit. In other words it is the most minimal structure to be found within the crystal that is duplicated many times in order to compose the crystal itself. Sometimes this is more than the biological unit. For example the structural information deposited for the MnSOD from *E.coli* contains seven protein subunits even though the biological unit is a dimer. In the case of MnSOD2, the asymetric unit is two subunits while the biologically active protein is tetrameric. Further manipulations are therefore required to arrive at the all-important biologically active form of the protein (Figure 9).

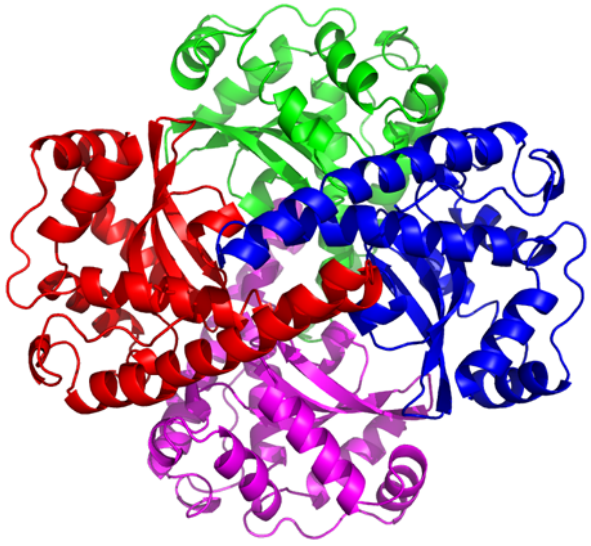

Figure 9: The biologically active three-dimensional structure of SOD-2, one of two MnSODs from *C.elegans* is homotetrameric. Each of the four chains is shown in a different colour. Only the protein backbone is shown in ribbon representation.

6.1 Conclusion

The determination of the structure of proteins is now considered to be the last hurdle to unlocking the molecular secrets of their mode of action. Over recent years, for example, the Center for Structural Genomics of Infectious Diseases (CSGID)(http://www.csgid.org/2014) and the Seattle Structural Genomics Center for Infectious Disease (SSGCID)(http://www.ssgcid.org/2014) have worked together to determine the structure of over one thousand proteins from forty pathogenic organisms responsible for diseases such as leprosy, cholera, TB and influenza. Other groups have obtained the structure of the HIV protein responsible of hijacking human cells (Tahirov et al., 2010). And recently scientists from Scripps Institute and Cornell School of Medicine have determined the structure of a number of G-protein-coupled receptors that are a vitally important component of many signaling pathways in many different types cells (gpcr.scripps.edu/2014) (Huang et al., 2013). Collectively these and similar breakthroughs are considered as important as the completion of the human genome sequence. The availability of this type of information for proteins marks the start of an new era in the advancement of science and medicine, enabling not only the understanding of protein function in health and disease but also providing opportunities for better diagnostic tools and development of novel drugs designed for specific targets.

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Research Article

A first record of *Chara vulgaris* var. *papillata* Wallroth. Ex A. Braun (Charales) in the Maltese Islands (Central Mediterranean)

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Abstract. The Maltese list of characeans includes *Chara vulgaris* of which the most commonly occurring variant is *C. vulgaris* var. *longibracteata*. A less common variant, *C. vulgaris* var. *papillata*, has been recorded for the first time locally in mats of *Chara vulgaris* examined from il-Qattara pool and Qawra (Dwejra, Gozo).

Keywords Stoneworth - *Chara vulgaris* var. *Papillata* - il-Qattara.

1 Introduction

Numerous freshwater wetlands found in the Maltese islands (ranging from small rockpools accumulating in karst depressions to freshwater wetlands and artificial reservoirs) are commonly colonised by macroscopic Charales. Locally these are represented by the genera *Chara*, *Tolypella* and *Nitella*. The following is the locally occurring species list (Lanfranco, 1969; Lanfranco, 2002):

- *Tolypella glomerata* (Desvaux) von Leonhardi 1863
- *Tolypella nidifica* (Muller) von Leonhardi 1857[1]
- *Chara vulgaris* Linnaeus 1753 *sensu lato*
- *Chara vulgaris* var. *longibracteata* (Kutzing) Groves et Bullock-Webster
  = *Chara vulgaris* var. *foetida* A. Braun forma *subinermis* A. Braun
  = *Chara foetida* A. Braun forma *subinermis* A. Braun β *longibracteata* A. Braun
- *Chara vulgaris* var. *gymnophylla* (A. Braun) Nyman
  = *Chara gymnophylla* A. Braun 1834
- *Chara globularis* Linnaeus var. *globularis* f. *globularis* Thuillier 1799
  = *Chara globularis* Thuillier 1799
  = *Chara fragilis* Desvaux
- *Nitella flexilis* (Linnaeus) Agardh 1824[2]

The following brief notes deal with the discovery of Chara vulgaris var. papillata in the island of Gozo. Material collected has been deposited in the herbarium of the author.

2 Methods

Material examined: Fresh material was collected from shallow areas of il-Qattara pool (Dwejra, Gozo: 36°3′3.47″N 14°11′32.41″E) in February 2005, where material persisted until May. Material was also collected from a watercourse in the Qawra area (36°3′6.44″N 14°11′31.78″E) at the same time, where the alga persisted until April when the watercourse dried. Token specimens collected from mats in the two locations were examined with the naked eye and under the stereomicroscope at magnifications ranging from ×7 to ×30.

Description of material: Thallus up to 50cm, greyish-green, highly encrusted. Cortex with a single secondary row between each primary row; papillate spine cells up to 1mm (visible with the naked eye) sticking out from the cortex. Cortex is aulocanthous.

3 Discussion and Conclusions

C. vulgaris is a variable species and a number of described varieties occur (Bryant and Stewart, 2002). The various forms may interchange over time both within the

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[1]This species is similar to T. glomerata, however possessing larger gametangia (Bryant and Stewart, 2002). (Lanfranco, 2002) attributes all records of T. nidifica to T. glomerata due to probable misidentification.

[2]This characeaen has never been observed after being recorded by (Gulia, 1877).

Figure 1: View of specimen habit (×7). Scale bar: 5mm.

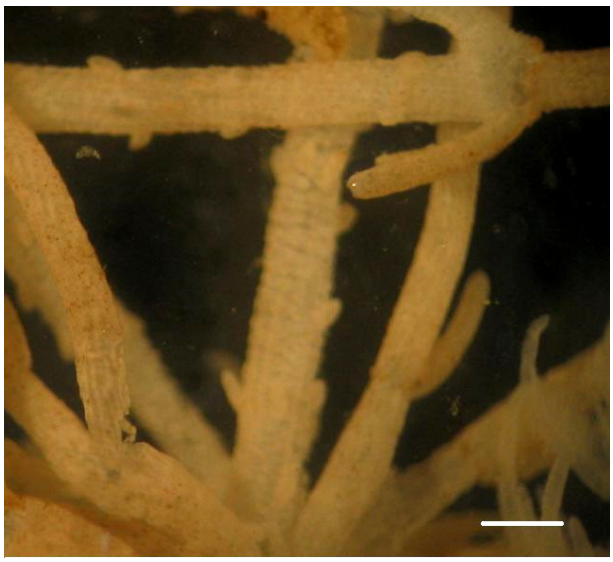

Figure 2: Detail of stems (×10). Note spine cells and encrustation. Scale bar: 1mm.

same population and within individuals. Different varieties may therefore represent ecomorphic expressions of the same species.

Within all varieties, spine cells are deciduous, persisting toward the apex of the branch. In the identified variety, the characteristic feature is the length of the spine cells (wider than the branches) which are also clearly deciduous (persisting only on the newer end of the stem). Spine cells have been variously described as either lying within the groove formed by the primary rows (Moore, 1986) or sticking out (Groves and Bullock-Webster, 1924), as is the case with the discovered specimens.

Il-Qattara is a permanent freshwater pool first described by (Schembri et al., 1987) and consequently by (Anderson and Schembri, 1989). More recently it was described by (Gauci, 1996) and (Camilleri, 2006) as being colonised by hydrophilic flora (including numerous algae) and fauna. (Camilleri, 2006) notes that salinity varies gradually, from 0.26 during the wet season to 1.35 during the warm season, confirming its freshwater characteristics. *C. vulgaris* var. *papillata* was recorded during the time of year where salinity ranged from 0.82 to 1.10. In contrast to il-Qattara pool, the watercourse in the Qawra area is ephemeral and exists through overspill from il-Qattara pool and runoff from surrounding higher ground and from Wied Għorof/Wied Merell, Wied Sufar and Wied il-Kbir (the latter also drains into il-Qattara pool). The watercourse desiccates once rain-induced runoff ceases. Therefore, it is evident that specimens in this area dry out due to lack of water availability. Conversely, the demise of specimens in il-Qattara pool may be attributed to a number of factors, of which gradual increases in salinity and temperature may play a role.

In both the il-Qattara and Qawra sites, specimens have been noted growing in dense mats within shallow water, mixed with *C. globularis*, *C. vulgaris* var. *longibracteata* and *Cladophora* spp. This observation is concordant with the findings by (Camilleri, 2006) for the month of May. The growth of mixed varieties of *C. vulgaris* within the same population is consistent with literature and indicative of ecomorphosis within the population. Extensive surveys of the pool and watercourse carried out in March 2009 noted only the *longibracteata* variety.

The finding of the *papillata* variety, restricted to one location within the island of Gozo is of significance to the Characean flora of the Maltese Islands. It may result from ambient conditions which allow increased plasticity within the population of *C. vulgaris* when compared with populations of the same species in other areas.

Acknowledgments

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Research Article

Phospho-Akt expression is high in a subset of Triple Negative Breast Cancer Patients

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Abstract. The most commonly used biomarkers to predict the response of breast cancer patients to therapy are oestrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2). Patients positive for these biomarkers are eligible for specific therapies such as anti-oestrogen therapy in the event of ER and PgR positivity, and trastuzumab, a monoclonal antibody, in the case of HER2 positive patients. Patients who are negative for all these three biomarkers, the so-called triple negatives, however, derive little benefit from such therapies. The PI3K/Akt pathway is activated in triple negative breast cancer cases, providing a possible target for therapy.

The activation of Akt was investigated in Maltese triple negative breast cancer cases using an antibody detecting Akt phosphorylated at serine 473 (anti-Akt pS473). The study showed that 26% of triple negative breast cancer patients had an elevated level of Akt (pS473). Furthermore, FTY720, a pharmacological activator of the phosphatase PP2A, was shown to block Akt activation at a concentration of 1μM, in HCC1937 cells subjected to insulin-like growth factor 1 (IGF-1).

Our data defined a subset of triple negative breast cancer patients based on high activity of AKT (pS473). This subset would be eligible for treatment using therapies which target the PI3K/Akt pathway, such as kinase inhibitors or phosphatase activators. In support of this, the BRCA1 mutant cells (HCC1937) were sensitive to the PP2a activator, FTY720. This suggests that FTY720 is a potential drug for use in adjuvant therapy in breast cancer cases having a high Akt (pS473).

Keywords Triple Negative Breast Cancer - biomarkers - phosphatases - Akt - BRCA1.

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1 Introduction

Breast cancer accounts for approximately 23% of cancer cases in females and is responsible for 14% of cancer-related deaths in females (Jemal et al., 2011). Classification of breast cancers is based on morphological features (lobular or ductal) and on the expression of the oestrogen receptor (ER), progesterone receptor (PgR) and the human epidermal growth factor receptor 2 (HER2). Breast cancer patients found negative for ER, PgR, and HER2 (triple negative) tend to fall within the subset of cases which have a basal-like phenotype and have a worse overall and disease-free survival (Onitilo et al., 2009). Interestingly, this subset has a higher occurrence of phosphatidylinositol 3-kinase (PI3K) pathway activation (Umemura et al., 2007).

Receptor tyrosine kinases such as Her2 and insulin-like growth factor 1 (IGF-1) receptor activate the PI3K pathway, initiating a cascade of signals. Active PI3K generates phosphatidylinositol 3,4,5 triphosphate (PIP3), which serves as an anchor for Pleckstrin homology (PH)-domain containing proteins, both adaptor molecules such as Gab2 and Dok1 and kinases such as Tec, Btk, PDK1 and Akt (Leevers et al., 1999; Saito et al., 2001; Stokoe et al., 1997; Tang et al., 1994). Activation of Akt increases cell cycle progression and maintains mammalian target of rapamycin

(mTOR) signalling resulting in enhanced cell proliferation and survival. The PI3K pathway is attenuated by phosphatases, including PTEN, which dephosphorylates PIP3 (Russillo et al., 2011) and PP2A, which inactivates mTOR effectors (Liu et al., 2010). In addition, inactivation of PP2A by phosphorylation at Tyrosine 307 is significantly correlated with HER2 positive tumour progression (Wong et al., 2010).

PI3K/Akt activates mTOR/Frap1 through phosphorylation of the tumour suppressor complex Tsc1/Tsc2 (tuberous sclerosis protein 1/2). Tsc1/Tsc2 releases Rheb (Ras-homolog enriched in brain), a small GTPase that positively modulates mTOR function (Inoki et al., 2003). Activation of mTOR results in phosphorylation and activation of ribosomal protein S6 Kinase (S6K; Rps6kb1; p70S6Kinase) and hierarchical phosphorylation of 4EBP (4E-binding protein) (Wang et al., 2005). Rapamycin-induced dephosphorylation of 4EBP is dependent on the activity of protein phosphatases type 1 and 2 (Chen et al., 1998), suggesting that mTOR inhibition releases a phosphatase to act on its downstream targets. Interestingly, studies on breast cancer cell lines show an increased sensitivity of triple negative cells to mTOR inhibitors (Noh et al., 2004). This suggests that deregulation of the mTOR effectors and/or regulators plays an important role in the pathology of triple-negative breast cancers.

Increased Akt activation correlates with low PTEN expression (Lopez-Knowles et al., 2010) and PI3K mutations (Stemke-Hale et al., 2008). Gain of function mutations in the PIK3CA gene (encoding the p110 catalytic subunit of PI3K) are present in 25% of invasive breast cancers (Bachman et al., 2004) and low PTEN expression in approximately 30% of invasive breast cancers (Tsutsui et al., 2005).

Loss of function of the phosphatases PTEN (Marty et al., 2008) and INPP4B (Gewinner et al., 2009) is associated with aggressive basal-like breast carcinoma. PTEN, INPP4B and PP2A are known antagonists of Akt phosphorylation, hence loss of phosphatase function leads to increased Akt activation. Interestingly, BRCA1 is known to activate PP2A, a phosphatase that dephosphorylates Akt at Threonine 308 (T308) and Serine 473 (S473) (Ma et al., 2007; Ugi et al., 2004). This is supported by the findings that loss of BRCA1 activity leads to increased Akt activity (Xiang et al., 2008) and reduced PP2A activity (Ma et al., 2007). In addition, BRCA1 is known to bind p-Akt and lead to its ubiquitination (Chen et al., 1998). In fact one finds an enhanced stability and higher expression of p-Akt in BRCA1 mutants, in which the mutant BRCA1 lacks the ability to bind to phosphorylated Akt (Xiang et al., 2008).

In vitro studies showed that serum starvation of phosphatase-depleted cells maintained a high pAkt signal (Fedele et al., 2010). In this study we tested the effect of PP2A activators on BRCA1 mutant cells which represent a subtype of triple negative breast cancer patients, potentially having a suppressed PP2A feedback mechanism. Interestingly, BRCA1 mutant cells (HCC1937) were sensitive to the PP2A activator, FTY720, resulting in enhanced dephosphorylation of Akt (pS473) upon 1 hour starvation. The ER positive cell line, MCF7, was less responsive to FTY720. In addition, restimulation of phosphorylated Akt (pS473) using IGF-1 was blocked by FTY720. A retrospective study of triple negative breast cancers showed a high activity of Akt (pS473) in 27% of the cases. These cases are eligible to pharmaceutical inhibition of the PI3K pathway and potentially activation of the phosphatase PP2A. Activation of PP2A will allow targeting of the deregulated PI3K pathway, including kinase mutants and cells with a low PTEN expression, but also BRCA1 mutants due to the sensitivity conferred by the lower PP2A activity.

2 Materials and Methods

Cell Lines Used and Culturing Conditions

Two adherent human breast cancer cell lines were used in the study: MCF-7 and HCC1937 (ATCC). MCF-7 and HCC1937 were both cultured in sterile T-25 flasks in an incubator with temperature set at 37°C, having an atmosphere of 5% CO_2 and 98% humidity. The MCF-7 cell line was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% Foetal Bovine Serum (FBS; Gibco, Invitrogen) and 1% Penicillin/Streptomycin (Gibco, Invitrogen). The HCC1937 cell line was cultured in RPMI-1640 (Sigma-Aldrich) containing 10% FBS and 1% Pen/Strep. Passaging was carried out when the cells reached around 90% confluence.

Dosage-Viability Experiments

The effect of different dosages of FTY720 (Cayman Chemical) on the viability of MCF-7 and HCC1937 cells was investigated before FTY720 was used in the experiments. MCF-7 and HCC1937 cells were seeded in 6-well plates and allowed to reach around 90% confluence. FTY720 was then added to each well such that the following concentrations were obtained: 0, 0.5, 1, 2.5, and 5 μM. These were incubated overnight (for 24 hours) and the percentage of viable cells measured. The percentage of viable cells was measured using a CASY Cell Counter and Analyser System (Roche).

Starvation and Starvation-Stimulation Experiments

MCF-7 and HCC1937 cells were seeded in a 96-well plate cultured for 3 days. The cells were first starved for 2 hours by serum deprivation. After the 2 hours elapsed, IGF-1 was added to selected wells to a final

concentration of 200ng/mL. Cells were stimulated for 30 or 60 minutes. FTY720 was added at a final concentration of 1μM. At the end of the procedure, the medium was removed from the wells by aspiration and the cells were fixed by the addition of 3.7% formaldehyde in 1xPhosphate Buffered Saline (PBS; Sigma-Aldrich) and an ICW assay was performed, using an AKt (pS473) antibody (Abcam).

Western Blotting (WB) Procedure
Western blotting was carried out on the cytoplasmic protein fraction of MCF-7 cells in order to confirm that the Akt (pS473) antibody to be used bound to the intended target specifically. The cytoplasmic protein lysate obtained from MCF-7 cells were separated using a 7.5% SDS-PAGE (Sodium Dodecyl Sulfate - PolyAcrylamide Gel Electrophoresis) procedure. Electrophoresis was run for 2 hours at 150V and 30mA. The separated proteins were then transferred using electroblotting (a semi-dry procedure) onto a nitrocellulose membrane using a tris-glycine transfer buffer. Blocking of the membrane was done for 1 hour at room temperature, using Odyssey blocking buffer (LI-COR Biosciences). The primary antibody was used at a dilution of 1 in 200 in blocking buffer and incubation was carried out at room temperature for 1 hour. The secondary antibody was an anti-rabbit antibody conjugated to IRDye680 (LI-COR Biosciences), and was used at a dilution of 1 in 15,000. Secondary antibody incubation was carried out for 45 minutes at room temperature. The membrane was then scanned using an Odyssey®Infrared Imaging System (LI-COR Biosciences), obtaining fluorescence at 700nm.

In-Cell Western (ICW) Assays
The LI-COR®In-Cell Western™Assay Kit was used in the ICW protocol. ICW assays were carried out on MCF-7 and HCC1937 cells after the starvation and starvation-stimulation experiments. Cells were first fixed (20 minutes in 150μl of 3.7% formaldehyde in 1x PBS per well) and then permeabilised (5 washes of 5 minutes each in 200μl of 0.1% Triton X-100 (Sigma-Aldrich) in 1x PBS per well). Blocking (for 1.5 hours with continuous shaking) was then performed using 150μl of Odyssey®blocking buffer per well. Primary antibody incubation was carried out using 50μl of a 1:100 dilution of Akt (pS473) antibody per well. Secondary antibody incubation was carried out for 1hr on a plate shaker at room temperature. The secondary antibody (anti-rabbit and conjugated to IRDye800) was used at a dilution of 1:800 (50μl per well) and was supplemented with two dyes which fluoresce at 700nm (DRAQ5 at 1:2000 and Sapphire700 at 1:1000). The plate was scanned using an Odyssey®Infrared Imaging System with detection in both 700nm and 800nm channels. The two dyes (DRAQ5 and Sapphire700) were added in order to be able to normalise the fluorescence obtained due to the Akt (pS473) to the number of cells present in a given well. This is important since variation in cell number could lead to inaccurate results.

Immunohistochemical (IHC) Staining of FFPE Tumour Samples
A total of 47 Formalin-Fixed Paraffin-Embedded (FFPE) triple negative breast cancer samples from 2001 to 2009 were chosen for use in the study. The FFPE material was subjected to immunohistochemical staining using the Vectastain Elite ABC kit (Vector Laboratories). 3μm sections were cut from the FFPE samples and placed on APES coated slides for IHC staining. FFPE tissue were dewaxed using xylene and rehdydrated in decreasing concentration of alcohol. No antigen retrieval was necessary in the procedure and endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide for 20 mins. Non-specific binding of the primary antibody was blocked by incubating the sections with diluted normal swine serum (DAKO - 1/20). The primary antibody used in the procedure (1/ 40) was a rabbit polyclonal antibody raised against Akt phosphorylated at Serine-473 (hereafter referred to as Akt (pS473)) (Abcam), where sections were incubated at 6°C overnight. After washing with PBS, the slides were incubated in diluted biotinylated goat anti-rabbit antibody (Dako - 1/200) for 60mins at room temperature. Following washing with PBS, the signal produced was amplified by incubating the sections with Avidin-Biotin Complex (ABC) containing horse radish peroxidase (HRP) for 60mins at room temperature. Visualisation was performed by the use of 3,3'-diaminobenzidine (DAB), a substrate of HRP which gives a permanent brown colour. Slides were counterstained with haematoxylin and dehydration was carried out in increasing concentration of alcohol. After clearing in xylene, slides were mounted with DPX. The stained sections were scored according to the intensity of the stain. The score ranged from 0 to 3, with 0 being negative and 3 being intense. Samples scoring 2 or higher were considered positive for the purposes of this study.

3 Results

In this study, BRCA1 mutant cells (HCC1937) were sensitive to the PP2a activator, FTY720. Re-stimulation of AKT (pS473) by IGF-1 was blocked by FTY720. A subset of triple negative breast cancer patients showed a high activity of AKT (pS473), with potential benefit for PI3K-pathway targeted intervention.

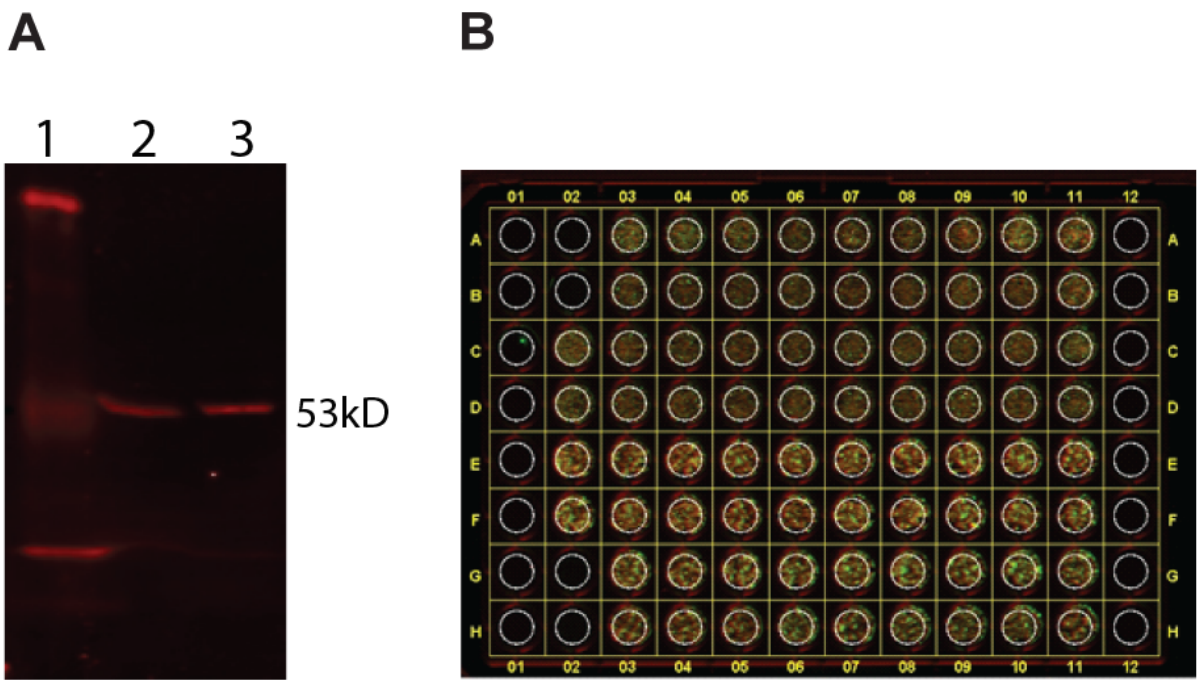

Figure 1: Specificity of Akt (pS473) staining and ICW analysis. A: Western Blot analysis staining using MCF-7 protein lysates stained for Akt (pS473). Lane 1 contains the ladder while lanes 2 and 3 contain the MCF-7 protein lysates. B: Cells were seeded in 96 well plates and stained according to the ICW protocol. Scanned image with rows A to D containing HCC1937 cells while rows E to H contain MCF-7 cells. Columns 3, 6, 9 contain cells which have been starved for 2 hours. Columns 4, 7, 10 contain cells which have been starved for 2 hours and stimulated for 30 minutes while columns 5, 8, 11 contain cells which have been starved then stimulated for 60 minutes. Column 2 contains the controls, A2, B2, G2, H2 being negative controls, while C2, D2, E2, F2 contain positive controls. Columns 1 and 12 are empty.

Akt (pS473) Antibody Staining is Specific

The specificity of the Akt (pS473) antibody to be used was first investigated by Western blotting carried out on cytoplasmic protein lysates taken from cultured MCF-7 cells. The Akt (pS473) antibody was expected to bind to phosphorylated Akt at a molecular weight of 56kDa. The bands visualised in lanes 2 and 3 are close to the band in the ladder (lane 1), which corresponds to a molecular weight of 60kDa (Figure 1A). This confirmed that the antibody indeed binds to the intended target and that it is quite specific since no other bands are visible on the blot.

Cell Viability Unaffected by FTY720

FTY720 is an activator of PP2A, a phosphatase known to inhibit Akt activity. Before performing the starvation and stimulation experiments, it was necessary to ensure that the FTY720 concentrations used in the study do not affect the viability of these cells. MCF-7 and HCC1937 cells were incubated for 24 hours in their respective mediums containing FTY720 at a concentration ranging from 0μM to 5μM. Concentrations of FTY720 used were 0, 0.5, 1, 2.5, and 5μM. This range of concentrations was chosen based on the fact that FTY720 causes significant growth inhibition of MCF-7 cells, when used in concentrations of 5μM or higher, over a period of 48 hours (Nagaoka et al., 2008).

The percentage of viable cells was found using a cell counter which is able to distinguish the viable cells from the dead cells, and hence give the viability as the percentage of cells in a suspension which are alive. From Table 1 one can note that FTY720 does not affect cell viability at the concentrations used since the percentage of viable cells remained at around 90% throughout. FTY720 was used at a concentration of 1μM in the experiments carried out.

Table 1: Percentage viability of cultured cells when exposed to a range of FTY720 concentrations over 24 hours. MCF-7 and HCC1937 cells remained viable up to a concentration of 5μM FTY720. FTY720 can be used to investigate phosphorylation events without affecting viability.

FTY720 Concentration (μM)	Percentage Viability (%)	
	MCF-7	HCC1937
0	91.92	91.23
0.5	90.87	90.50
1	89.72	91.45
2.5	90.95	92.49
5	91.68	91.39

Serum Deprivation Results in Akt (pS473) Dephospho-

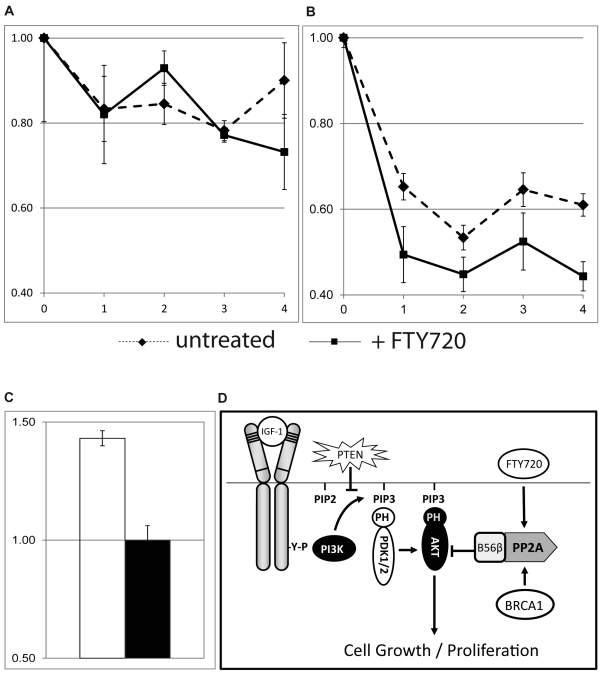

Figure 2: HCC1937 are sensitive to serum starvation and FTY720 addition. Cells were serum starved for 4 hours in the presence (solid line) or absence (dashed line) of 1μM FTY720. Fold change in Akt (pS473) during starvation was measured for the MCF-7 (A) and HCC1937 (B) cell lines. Each point represents a mean taken over three values, with the error bars representing the standard error. C: Following 2 hours starvation, HCC1937 cells were stimulated for 1 hour with 200ng/mL IGF-1 in the presence (black bar) or absence (open bar) of 1μM FTY720. D: Schematic diagram showing the attenuation mechanism of the PP2A holoenzyme complex. BRCA1 activates PP2A activity, hence supporting the sensitivity of the BRCA1-mutant HCC1937 cells to FTY720 (which is an activator of PP2A activity).

rylation

During the starvation experiment the phosphorylated Akt (pS473) level was suppressed in MCF-7 and HCC1937 cells (Figure 2A, B). In order to better analyse the observed starvation pattern, the data was subjected to the ANOVA statistical test at a significance level of 0.05. It was found that there is a statistical difference between the mean values obtained in the case of both MCF-7 ($p=0.011$) and HCC1937 ($p=0.000$), showing that starvation significantly affected the level of Akt (pS473). In particular, it decreases significantly in the first hour ($p=0.017$ for MCF-7, and $p=0.000$ for HCC1937) but any changes beyond the first hour were not statistically significant. Dephosphorylation of Akt in HCC1937 was stronger, indicating that the BRCA1 mutant cells are more sensitive to starvation.

The use of FTY720 at a concentration of 1μM resulted in the same pattern: a statistically significant decrease in Akt (pS473) occurred within the first hour ($p=0.005$ for MCF-7, $p=0.000$ for HCC1937). FTY720 did not produce a statistically significant difference in Akt (pS473); however, in the case of HCC1937 the level of Akt (pS473) was slightly lower when FTY720 was used compared to starvation in the absence of FTY720 (Figure 2B).

IGF-1 stimulation of p-Akt is suppressed by FTY720. From the previous starvation experiment a 2 hour starvation time point was selected since during starvation p-Akt decreases significantly during the first hour. MCF-7 and HCC1937 cells were first subjected to starvation (2hrs) followed by stimulation with 200ng/mL IGF-1 (a known stimulator of Akt) for 30 or 60 minutes. FTY720 was also used at a concentration of 1μM to investigate whether this affects the stimulation.

The effect of FTY720, on Akt activation (measured as the level of phosphorylated Akt (pS473)) in the two human breast cancer cell lines (MCF-7 and HCC1937) under conditions of starvation and stimulation was investigated. ICW assays were carried out to measure the level in Akt (pS473) in these cells which were either starved by serum deprivation, or stimulated using IGF-1 following a period of starvation. The Akt (pS473) antibody used in IHC analysis was also used in the ICW assays. Akt (pS473) in HCC1937 increased by a 1.4 fold change, upon IGF-1 stimulation, when compared to the level at 2 hrs starvation. The addition of 1μM of FTY720 abrogated Akt activation (Figure 2C). Stimulation of MCF-7, following starvation, was marginal (fold change < 1.2) and hence the effect of FTY720 on stimulation was not possible (data not shown).

Akt (pS473) Activity is High in a Subset of Triple Negative Breast Cancer Patients

Triple negative breast cancer samples were analysed using immunohistochemistry to investigate the fraction which is characterised by elevated Akt activation. Of the 47 chosen triple negative FFPE samples, 2 samples could not be adequately analysed by immunohistochemistry due to insufficient amounts of tumour tissue. The other 45 were successfully stained with the Akt (pS473) antibody. The staining pattern obtained with the p-Akt antibody was, as expected, cytoplasmic and granular in appearance (Figure 3). The staining intensity from sample to sample varied and a range of scores were obtained from the triple negative breast cancer samples.

Triple negative breast cancer samples having an intensity score of 0 or 1 were considered as having a low expression of Akt (pS473) and consist of 73% of analysed samples. Samples having an intensity score of 2 or 3 were considered to have a high level of Akt (pS473). This subset accounts for 27% of stained triple negative breast cancer samples.

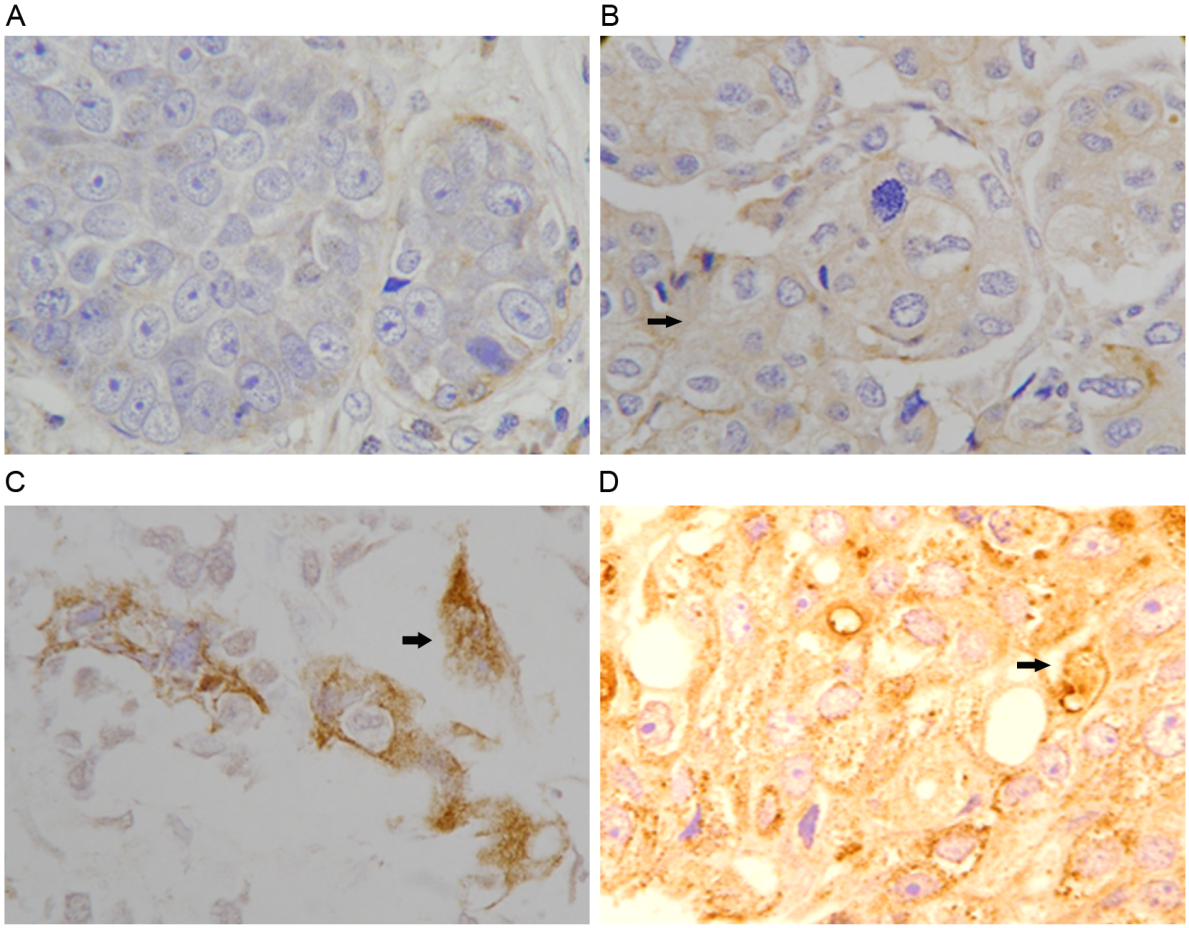

Figure 3: FFPE breast cancer patient sample sections stained with the Akt (pS473) antibody, showing different staining intensities. In all cases magnification was by a factor of 600, and arrows point towards a region of tumour with the appropriate intensity of stain. A: Intensity=0 (Negative); B: Intensity=1; C: Intensity=2; D: Intensity=3.

4 Discussion

This study revealed that a subset of triple negative breast cancer samples stained high for Akt (pS473). FTY720, a pharmacological activator of PP2A, was shown to abrogate Akt activation by IGF-1, in the BRCA1 mutant cell line, HCC1937.

IGF-1 Stimulation of p-Akt abrogated by FTY720
The effect of FTY720 on Akt activity in the human breast cancer cell lines, MCF-7 and HCC1937 was investigated. The HCC1937 cell line is negative for ER, PgR, and HER2 and bears mutant PTEN and BRCA1 (5382insC mutation) genes. One would anticipate that the BRCA1 mutant cells, HCC1937, have a low PP2A activity. This is based on the studies showing that knockdown of the BRCA1 gene leads to reduced PP2A activity (Ma et al., 2007). In the case of MCF-7, there is a significantly lower expression of PP2A Aα subunits (Suzuki and Takahashi, 2003). Lowered expression of this subunit is associated with enhanced activation of Akt with higher expression of Akt (pS473) (Chen et al., 2005).

MCF-7 and HCC1937 cells were subjected to starvation in the form of serum deprivation followed by stimulation using IGF-1 (at 200ng/ml), both in the presence and absence of FTY720. IGF-1 was chosen as a stimulant since it is known to increase Akt activity by phosphorylation at T308 and S473 (Alessi et al., 1996). FTY720 is a pharmacological activator of PP2A, and was used to investigate its effect on Akt activity during serum deprivation and IGF-1 stimulation in the MCF-7 and HCC1937 cell lines. PP2A is known to inhibit Akt activity by inhibiting phosphorylation at both T308 and S473 (Rodgers et al., 2011). Figure 2D shows the pathway involved in the starvation-stimulation including the action of FTY720. FTY720 was used at a concentration of 1μM since it was shown to significantly inhibit growth in MCF-7 cells at concentrations of 5μM and higher (Nagaoka et al., 2008).

FTY720 at a concentration of 1μM had minimal effect during starvation. In the case of HCC1937, a lower Akt

(pS473) level was observed when FTY720 was added; however, this was not statistically significant except at 4 hours of starvation. In the case of MCF-7 there was almost no difference in Akt (pS473) activity with and without FTY720 during starvation. FTY720 had a considerably more pronounced effect during IGF-1 stimulation, where it suppressed Akt (pS473) stimulation in the HCC1937 cell line. Of particular interest is the fact that the HCC1937 cell line is triple negative and is known to have an enhanced Akt activity. This implies that FTY720 is a potential therapeutic agent in the treatment of triple negative breast cancer patients having an elevated Akt activity. This is especially important since there are few therapies which are effective in treating triple negative breast cancers.

A Subset of Triple Negative Breast Cancers Associated with High Akt (pS473)
To investigate the activation of Akt among triple negative (ER, PgR, and HER2 negative) breast cancer cases, a number of triple negative FFPE samples were chosen and stained with the Akt (pS473) antibody. Phosphorylation of Serine 473 and Threonine-308 are required for full activation of Akt (Bellacose et al., 2005). A wide range of scores were obtained (Table 2), with 16% (7/45) samples showing no staining, 58% (26/45) showing weak staining (score 1), 22% (10/45) showing intermediate staining (score 2), and 4% (2/45) showing strong staining (score 3).

One could split the group of triple negatives into two subgroups: those that had a low Akt (pS473) score (0-1, 74%) and those that had a high Akt (pS473) score (2-3, 26%). The subset of triple negatives having a high activation of Akt would be eligible for therapy targeted at the PI3K/Akt pathway. This is of great importance since triple negative breast cancer patients derive little benefit from current therapies.

Table 2: Distribution of Intensity scores following Immunohistochemistry staining with Akt (pS473). The range of Akt (pS473) scores and the respective frequency of each among triple negative breast cancer (TNBC) FFPE samples. 45 samples were stained in all. Intensity of pAkt staining is high in 12/45 (26%) of TNBC patients.

Intensity Score	Frequency
0	7
1	26
2	10
3	2

5 Conclusion

This study has shown that a subset of triple negative breast cancer (TNBC) cases in Malta, consisting of 26% of cases, have a moderate to high activation of Akt. Staining for phosphorylated Akt can be introduced in the clinic to classify TNBC patients and predict the potential use of targeted therapies. This subset would be eligible for therapies targeting the PI3K/Akt pathway. This is of great importance due to a current lack of effective therapies against triple negative breast cancer cases. Potential therapies which target the PI3K/Akt pathway include kinase inhibitors of mTOR and Akt, such as Palomid529 (Xue et al., 2008), and phosphatase activators such as FTY720 (Ugi et al., 2004). The potential use of FTY720 as a therapy in breast cancer cases having elevated levels of Akt (pS473) is supported by the finding that at a concentration of 1μM it suppressed stimulation of Akt activity by IGF-1 in HCC1937 cells *in vitro*. Interestingly, a correlation between a high level of Akt (pS473) and low expression of BRCA1 was found in Breast Cancer patients (Xiang et al., 2011). This supports our observations that breast cancer cases bearing a mutant BRCA1 gene or reduced BRCA1 expression could also be eligible for therapy targeting the PI3K/Akt pathway.

Competing interests

The authors declare that they have no competing interests.

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Research Note

Does the absolute HbA_{1c} improve the genotype-phenotype association in Type 2 Diabetes?

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Abstract. HbA_{1c} is a measure of the mean blood glucose levels for the prior 90 - 120 days, the mean lifetime of red blood cells. However, factors that influence the erythrocyte turnover or the biochemical structure of haemoglobin (Hb) might complicate the interpretation of results. With a frequency of haemoglobinopathies of around 5% in the Maltese population, an alternative biomarker should be considered. The aim of this study was to determine whether the absolute HbA_{1c} could improve the genotype-phenotype association in Type 2 Diabetes Mellitus (T2DM) and whether it could thus be an alternative measure.

Ion-exchange high performance liquid chromatography (HPLC) and polymerase chain reaction (PCR) were used to genotype and phenotype five different groups of subjects: haematologically normal adult controls, anaemics (Hb<10g/dL), β-thalassaemics, normal pregnant women and type 2 diabetics (controlling their diabetes either by diet alone, or using metformin for up to six months). The single nucleotide polymorphisms (SNPs) selected were in the ADRB2, LEP, FABP2, TCF7L2, MIF, IL6 and UCP1 genes.

Statistical analysis showed that the absolute HbA_{1c} did not improve the genotype-phenotype association, as it showed the same trends as the relative HbA_{1c}. The difference between the HbF and HbA_{1c} is due to the homogenous distribution of HbA_{1c} among erythrocytes, unlike HbF. *In vitro* glycation showed that Hb Beta-Valletta, found in 1.8% of Maltese adults, does not influence glycation and thus the HbA_{1c} is not influenced by this variant in heterozygotes/ homozygotes.

Keywords HbA_{1c} - Hb variants - HPLC - diabetes - SNPs - genetics.

Hb: haemoglobin
T2DM: Type II Diabetes mellitus
HPLC: High-performance liquid chromatography; RP-HPLC: Reverse phase- HPLC
Polymerase chain reaction
SNPs: Single nucleotide polymorphisms
DM: Diabetes mellitus
WHO: World Health Organisation
PBS: Phosphate-buffered saline
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1 Introduction

Diabetes mellitus (DM) is the most common serious metabolic disorder worldwide (Berg et al., 2006). According to WHO , around 2.8% of the global population currently suffer from type 2 diabetes mellitus (T2DM) and this value is expected to rise to around 4.4% in 2030 (Sicree and Shaw, 2007).T2DM is a complex disorder that may be considered an intermediate step in the progression from glucose intolerance to pre-prandial hyperglycaemia and macro- and micro-vascular complications (American Diabetes Association, 2012; Bennett et al., 2007). It is a heterogeneous disorder that is influenced by both genetic and environmental/ lifestyle factors, such as obesity and age (Olefsky, 2001).

The need for testing is important as preventative measures may decrease the risk of developing T2DM, or in the least retard the development of cardiometabolic complications (Diabetes Control and Complications Trial Research Group, 1993). The HbA_{1c}, a measure of the mean glucose concentration over the lifetime of the red blood cell (90-120 days on average) (Bennett et al., 2007), is commonly used to diagnose T2DM. Its suitability stems from its independence from prandial status, diurnal fluctuations and exercise (Sacks, 2003).

HbA_{1c} is formed by the process of non-enzymatic glycation: condensation of glucose to the free amine group of the N-terminal valine on the β-chain followed by the Amadori rearrangement (Bunn et al., 1976; Peterson et al., 1998; Saudek et al., 2006; Schulz, 2006; Sicree and Shaw, 2007). The accuracy of the HbA_{1c} may be influenced by factors that have an effect on the structure and half-life of red blood cells (Bernstein, 1980; de Boer et al., 1980; Eberentz-Lhomme et al., 1984; Fluckiger et al., 1981; Hanson et al., 1983; Horton and Huisman, 1965; Huisman et al., 1983; Lind and Cheyne, 1979; Paisey et al., 1986; Phelps et al., 1983; Starkman et al., 1983; Tran et al., 2004). With a 2% prevalence of thalassaemia and around 5% prevalence of haemoglobinopathies and red blood cell disorders in the Maltese Islands (Felice, 2012), it is important to consider other indicators of hyperglycaemia, such as the absolute HbA_{1c}, that may be calculated simply and non-invasively or quantified directly by immunoassay or mass spectrometry. This was the objective of this study.

2 Methods

Ethics approval for this study was obtained from the University Research Ethics Committee of the University of Malta.

Phenotyping: The HbA_{1c}, HbA_2 and HbF of 62 haematologically normal adults (N), 95 haematologically normal pregnant women (P), 61 β-thalassaemics (T), 39 severe anaemics (A) and 100 type 2 diabetics (D) (on limited or no treatment) were determined using ion-exchange HPLC (Bio-Rad Beta-thalassaemia short program kit on Bio-Rad VARIANT) within one week of whole blood collection. The haemoglobin concentration was obtained from the CBC. The absolute HbA_{1c} was calculated as follows (Sinha et al., 2012):

$$\text{Absolute Hb } A_{1c} = \frac{\text{HbA}_{1c}(\%) \times [Hb]}{100}$$

Genotyping: DNA from the aforementioned samples together with 200 random neonates (C), was extracted using a salting out procedure. PCR (simple, allele-specific and tetra-arms PCR) was used to genotype the following SNPS:rs1042713 (ADRB2), rs7799039 (LEP), rs1799883 (FABP2), rs7903146 (TCF7L2), rs755622 (MIF), rs603573 (IL6), rs1800592 (UCP1) (Abou-Hussein, 2009; Al Ashtar, 2008).

In vitro glycation: 5 normal samples and 3 blood samples from Hb Beta-Valletta heterozygotes were incubated with varying concentrations of glucose (0mM to 100mM in 10mM increments) in phosphate-buffered saline (PBS). The mixtures were incubated at 37°C for two hours prior to analysis using ion-exchange HPLC (Bio-Rad Beta-thalassaemia short program kit on Bio-Rad VARIANT).

In another experiment, 2 normal adult blood samples were incubated with 0mM, 50mM and 100mM glucose in PBS solutions for two hours at 37°C in a water bath. These were then analysed by reverse phase (RP)HPLC (Bio-Rad VARIANT with external JASCO UV-975 UV/Vis detector set at 215nm).

Data Analysis: Descriptive analysis, One Way Anova, scatter diagrams and correlations were carried out using IBM SPSS v17.0.Allele frequencies were calculated as follows:

$$p = f(AA) + \frac{1}{2} f(Aa)$$

$$q = f(aa) + \frac{1}{2} f(Aa) = 1 - p$$

3 Results

Phenotyping: As seen in table 1, diabetics and pregnant women exhibited the greatest and lowest values respectively, with normal adults closely following the latter. Thalassaemics and anaemics exhibited intermediary values with anaemics giving somewhat higher values.

Table 1: Values of glycation.

		HbA_{1c} (%)	Abs HbA_{1c}(g/dL)	HbA_{1c}/total HbA	Hb A_{1c}/(HbA_0 + HbA_{1c})
N	Avg.	4.88	0.66	5.06	5.31
	Std. Dev.	0.50	0.10	0.83	0.55
P	Avg..	4.38	0.53	4.62	4.77
	Std. Dev.	0.42	0.11	0.45	0.47
T	Avg.	5.54	0.61	5.99	6.29
	Std. Dev.	1.19	0.22	1.37	1.42
A	Avg.	6.12	0.64	6.44	6.62
	Std. Dev.	1.74	0.23	1.85	1.88
D	Avg.	8.14	1.14	/	/
	Std. Dev.	3.56	0.54	/	/

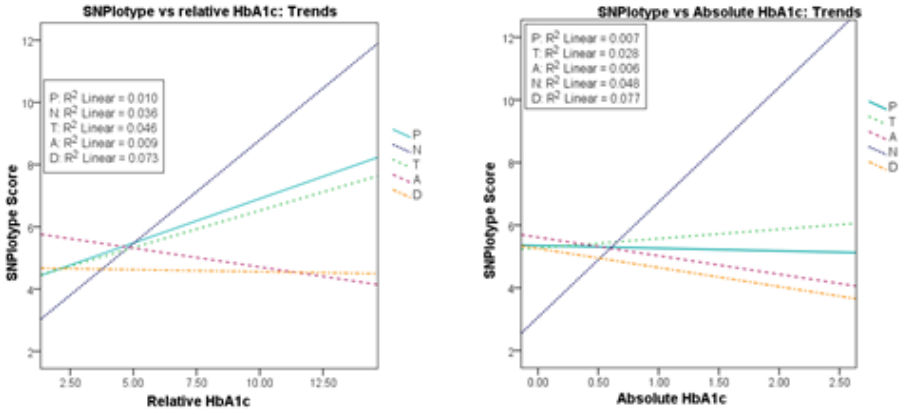

Figure 1: SNPlotype plots for the trends of the relative HbA_{1c} and the absolute HbA_{1c}.

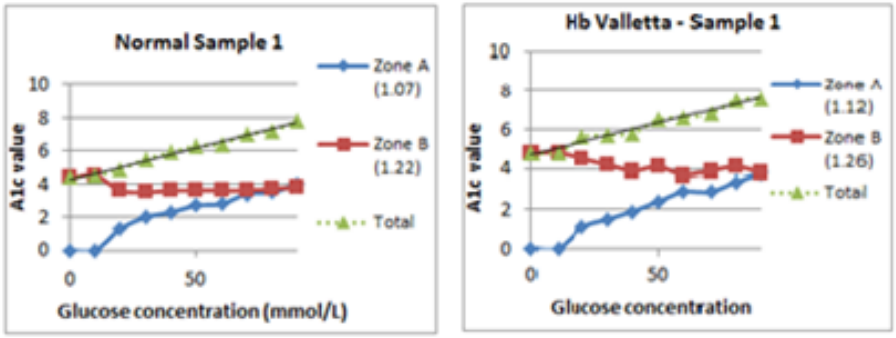

Figure 2: Overview of *in vitro* glycation results.

Diabetics exhibited a mean significant difference compared to all the other groups for both the relative and absolute HbA_{1c} at the 0.05 level and was the only significantly different group when considering the mean absolute HbA_{1c}.

SNPlotypes: The trend noted for normal adults (Fig. 1) was a proportional increase in glycation and genetic risk (as denoted by an increase in SNPlotype). This trend was not seen for the other groups. An inversely proportional relationship was observed for diabetics (Fig. 2).

Allele frequencies and correlations: These varied from one group to the next, with significant differences shown between cords and normal adults. Significant correlations were noted for the HbA_{1c} with MIF in pregnant women and ADRB2 in diabetics. No significant correlations were observed for the absolute HbA_{1c}.

Invitroglycation: On increasing the glucose concentration for both normal adult and Hb Beta-Valletta samples, the zone representing the HbA_{1c} (zone B in Fig. 2) was seen to increase in area. When the glucose concentration was increased above normal physiological conditions, zone A was eluted and increased with increasing concentrations. The appearance of this zone was accompanied by an initial decrease in the HbA_{1c} percentage. RP-HPLC indicated that this new zone was not the result of modification to the α- and β-globin structures.

4 Discussion

The absolute HbA_{1c} enabled a better distinction between the different groups based on phenotype alone but it did not offer an improved association with the genotype. Glucose is circulated uniformly throughout the blood resulting in equal exposure of all haemoglobin to extracellular glucose. HbA_{1c} is distributed evenly throughout the blood since it is not influenced by selective survival. The absolute HbA_{1c} does not improve the genotype-phenotype association and both the relative and absolute HbA_{1c} are equally useful measures of glycation.

The different trends obtained indicate that the hyperglycaemic state noted in diabetics was not attributed to genetic risk but to lifestyle choices such as obesity and poor diet. In fact, a significant proportion of Maltese individuals are known to be overweight or obese (Savona-Ventura, 2001).

The allele frequencies show the importance of choosing the correct control population. Random cords represent the general population while the normal adults represent

individuals with normal levels of glycation and normal haemoglobin physiology. Thus, both types of controls were needed for this study.

Haemoglobin variants, such as Hb Marseille, influence the measurement of glycated haemoglobin. Incubation with glucose indicated that, although no hyperglycaemic Hb Beta-Valletta individuals were encountered in the laboratory, this variant does not influence the HbA_{1c} value.

The elution of Zone A may indicate that non-enzymatic glycation occurred at more than one site. Apart from the N-terminal valine, five lysine residues are available for glycation on the β-globin subunit. These are not glycated in HbA_{1c}. The earlier elution time of Zone A, indicating a greater negative overall charge, and the decrease of HbA_{1c} with increasing abundance of the new zone suggest that the positively charged lysine residues are undergoing non-enzymatic glycation.

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Review Article

Control of globin gene expression by Kruppel-like Factors

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Abstract. Kruppel-like factors (KLFs) are a family of seventeen proteins designated KLF1 to KLF17. KLFs are transcriptional factors that bind GC-rich sequences such as *CACCC* elements. The DNA binds to KLFs via three carboxyl-terminal Cys-2/His-2 zinc fingers. KLFs control cell differentiation and embryonic development. They are also implicated in a number of cellular functions such as erythropoiesis, proliferation and tissue development. This review will focus primarily on KLFs that are involved in haemoglobin control. These include KLF1, KLF2, KLF3, KLF8 and KLF10. The connection between human KLF1 and elevated foetal haemoglobin was first identified in a study done by (Borg et al., 2011) on a large Maltese family with Hereditary Persistence of Foetal Haemoglobin (HPFH) where a nonsense mutation in the Erythroid Kruppel-Like Factor 1 gene (*KLF1*) was identified as the main cause of HPFH. KLF2 is a positive regulator of mouse and human embryonic β-globin genes and it overlaps with KLF1 in embryonic erythropoiesis. KLF3 and KLF8 expression is driven by KLF1 while together KLF3 and KLF8 participate in the silencing of embyronic globin expression during development. KLF10 expression was also shown to be associated with high foetal haemoglobin levels in βthalassaemia patients undergoing hydroxyurea treatment.

Keywords Haemoglobinb - Kruppel-like factor 1 - β-thalassaemia - Erythropoiesis.

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1 Introduction

The study of haemoglobin (Hb) switching in humans has provided a focus in haematology due in large part to fundamental importance of gene switching in human biology and the clinical significance of the foetal to adult globin switch for developing targeted methodologies to for the treatment of the β-type haemoglobin maladies such as thalassaemia and sickle cell disease (Orkin and Higgs, 2010). Hb is constituted of two α-like and two β-like globin chains, encoded by genes in the *HBA* and *HBB* loci, respectively. Developmental regulation of globin genes results in expression of stage-specific Hb molecules.

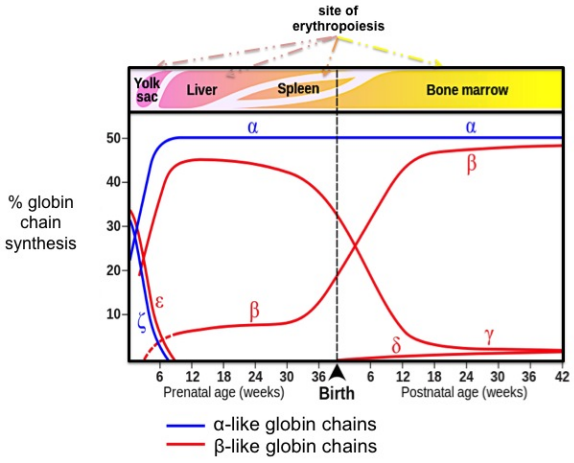

Figure 1: **Developmental Globin Chain Synthesis.** The timeline of the expression of the human globin genes is shown on the X-axis.

2 Developmental Control of Globin Gene Expression

The exact nature of globin gene switching from ɣto βis still largely unknown and it is through very specific ques-

tions that one is able to answer parts of this occurrence. Hereditary persistence of foetal haemoglobin (HPFH) has been long sought as a useful contributory 'condition' that can yield additional information on how foetal haemoglobin (Hb F) is regulated and controlled *in vivo* at the adult stage. Hb F ameliorates the symptoms of β-thalassaemia and sickle cell disease (SCD), and reactivation of the *HBG1/HBG2* genes in adults is therefore of substantial interest for the clinical management of β-type haemoglobin disorders. During the development of the human body, seven normal haemoglobin types are expressed in developmental pattern. The production of normal haemoglobin is characterized by two switches (Fig. 1). These are, the embryonic to foetal haemoglobin switch and the foetal to adult haemoglobin switch, the latter being the subject of many critical questions and reviews (Bank, 2006). The embryonic haemoglobin is composed of Hb Gower 1 (α2ε2), Hb Gower 2 (ζ2ε2) and Hb Portland (ζ2γ2). As the expression of ζ- and ε- globin begins to cease after the first two months of gestation, the first haemoglobin switch occurs giving rise to the synthesis of HbF ($\alpha 2^{G}\gamma 2$) and ($\alpha 2^{A}\gamma 2$). The site of erythropoiesis also changes from the yolk sac and para-aortic region to the foetal liver (Dover and Boyer, 1980). HbF has a higher oxygen affinity since it binds 2,3-bisphosphoglyceric acid (2,3-BGP) less strongly predominates in the last two trimesters of gestation before the second globin switch takes place (Forget, 1998). The second globin switch which occurs at the time of birth, involves the decline of HbF synthesis coupled with increased synthesis of adult haemoglobin composed of HbA (α2β2) with a minor HbA2 (α2δ2) (Brinkman and Jonxis, 1935; Weinberg et al., 1983). This switch is also accompanied by a change in the site of erythropoiesis from the foetal liver and spleen to the bone marrow. However, the anatomical transitions are not thought to be the cause of the gene switching events.

Residual amounts of Hb F continue to be synthesized throughout adult life and expressed by F-erythrocytes (Hosoi, ; Stamatoyannopoulus and Grosveld, 2001). In the majority of adults, Hb F consists of less than 2% to total Hb, but there is considerable variation (Thein et al., 2009). Genetic studies have revealed at least three loci that could control Hb F levels in adults: *HBB* (11p15.4) (Gilman and Huisman, 1985; Craig et al., 1996), *HBS1L-MYB* (6q23.3) (Close et al., 2004; Craig et al., 1996; Garner et al., 1998) and BCL11A (2p16.1) (Menzel et al., 2007; Lettre et al., 2008). Together, these loci account for less than 50% of the variation in Hb F, indicating that additional loci may be involved. The HBB locus in particular contains an important promoter sequence variation at position -158 five primer (5') to the Gγglobin gene, called *Xmn1* site and other globin gene rearrangements (such as Gγ- Gγ, Aγ- Aγor multiple γglobin genes) that have shown to effect Hb F levels and Gγ:Aγratios in normal individuals and/or individuals with anaemic stress (Thein et al., 2009).

Clinical syndromes such as the β-thalassaemias, hereditary persistence of foetal haemoglobin (HPFH) and δβ-thalassaemias have all proved to be unique clinical models underlying the pathophysiology of globin gene expression and control in haemoglobinopathies. Genetic analysis of HPFH families is a particularly powerful approach to identify novel modifiers of Hb F levels (Close et al., 2004).

Indeed early clues (Weatherall and Clegg, 1975; Huisman et al., 1975) had indicated that important DNA regulatory sequences are present in between the foetal γglobin genes and adult βglobin genes. The various types of γglobin gene re-arrangements and deletional HPFH encompassing DNA regions of the Aγ-δintergenic region characterized this. A poly-pyrimidine rich region (PYR) is located in this intergenic region and attracts numerous protein complexes that together form a repressor-like complex that silences the foetal γglobin genes (Bank, 2006). A subset of important and critical genes that act in concert with other molecules and bring about control and regulation of haemoglobin are the Kruppel-like factor genes, most notably Kruppel-like factor 1 (KLF1), Kruppel-like factor 3 (KLF3) and Kruppel-like factor 10 (KLF10).

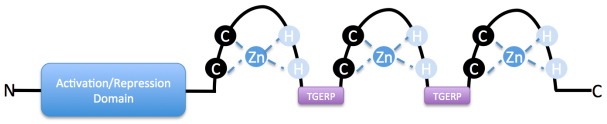

Figure 2: **Schematic diagram of the KLF1 molecule.** The three C-terminal C2H2 zinc fingers are shown, each chelating a single zinc ion. The fingers are linked together by "TGERP"-like motif, which assists in binding to target DNA. The activation/repression domain is found at the N-terminus of the molecule (Adapted from (Pearson et al., 2011))

3 Kruppel-like factor 1

Kruppel-like factors are a subset of genes that code for transcription factors designated KLF1 to KLF17 important in transcriptional regulation and control of a number of other genes. KLFs are implicated in many cellular functions such as erythropoiesis, cell differentiation, proliferation and tissue development (McConnell and Yang, 2010). A closely-knit network of KLFs interacts together to regulate the switch from foetal to adult haemoglobin. A duplicated *CACCC* sequence, known to attract and bind Kruppel-like factors, in the β-globin gene is located between -90 to -105 and is also present in the εand γgenes (Nienhuis and Maniatis, 1987). The α-globin gene promoter (Liebhaber et al., 1980) also comprises a *CACCC* similar sequence box of the β-globin promoter at position -84 to -89. The *CACCC*-box sequences (*CCACACCCT*) (Donze et al.,

1995) are frequently found in erythroid-specific gene promoters. Two such sites are present in the human and mouse β-globin promoters. KLF1 (Figure 2) is active in primitive and definitive haematopoiesis and not required for yolk sac erythropoiesis and erythroid commitment (Nuez et al., 1995; Perkins et al., 1995), suggested that it is important for the transition from foetal to adult globin expression in humans. Additionally, single base substitutions in the KLF1 binding sites in the β-globin gene promoter cause β-thalassaemia (Orkin et al., 1982). (Schoenfelder et al., 2010) found that mouse *Hbb* and *Hba* associate with hundreds of active genes from nearly all chromosomes in nuclear foci known as 'transcription factories'. The 2-globin genes preferentially associated with a specific and partially overlapping subset of active genes. (Schoenfelder et al., 2010) also noted that expression of the *Hbb* locus is strongly dependent upon KLF1, while expression of the *Hba* locus is only partially dependent on KLF1. Immunofluorescence examination of mouse erythroid cells displayed that most KLF1 concentrated to the cytoplasm and that nuclear KLF1 was present in isolated sites as clusters. Erythroid cells from KLF1 *null* mice specifically showed a disruption of the association of KLF1- regulated genes within the *Hbb*-associated network. KLF1 knockout more insipidly disrupted interactions within the specific *Hba* network. (Schoenfelder et al., 2010) revealed that KLF1-regulated genes share KLF1-containing transcription factories and that KLF1 is required for the clustering of these co-regulated genes. It was suggested that transcriptional regulation involves a complex 3-dimensional network rather than factors acting on single genes in isolation.

The description of the Active Chromatin Hub (ACH) gave a 3D picture of the human and mouse β-globin loci (Tolhuis et al., 2002; Palstra et al., 2003; Patrinos et al., 2004) and revealed a dynamic structure that communicates enhancers, promoters and specific regulators and co-regulators to execute gene transcription. In this model, the intervening sequences and non-transcribed genes are looping out of the active site of transcription, supporting a looping model for transcriptional activation of the globin genes.

The first transcription regulator that was shown to influence the formation of the ACH to execute correct expression of genes found within the β-globin locus was KLF1. In the absence of KLF1, a fully functional ACH cannot be formed (Drissen et al., 2004). Together with the observation that in KLF1 knockout mice loss of 5'HS3 and βmaj-promoter chromatin accessibility (Wijgerde et al., 1996) occurs, this implied that KLF1 is crucial for hypersensitive site formation and involvement of the Locus Control Region (LCR) and the β-globin promoter in the ACH, possibly through interactions with a SWI/SNF chromatin remodeling complex (Armstrong and Emerson, 1998). GATA1 was also shown to be essential for LCR-gene contacts (Vakoc et al., 2005) in contrast with Ctcf that was found to be dispensable for such interactions and globin gene expression (Splinter et al., 2006). A conceivable role of the LCR loop formation in RNAPolII loading to the promoters of the globin genes has been proposed (Johnson et al., 2001). Still, in KLF1 knockout erythrocytes, RNAPolII is loaded on the promoter of β-globin but the levels of Ser2 phosphorylated PolII, as a mark of active transcription, are reduced. This explains the decrease in β-globin expression (Bottardi et al., 2006). Thus, it is more likely that the recruitment of RNAPolII, at least to the β- globin promoter, is LCR independent while the transition from the initiation to the elongation step of active transcription is KLF1-dependent LCR formation (Sawado et al., 2003).

Mutations in human KLF1 were first reported by (Singleton et al., 2008) where it was found that 9 different loss-of-function KLF1 mutations were responsible for a rare In(Lu) blood group. One of the mutations results in a loss of 1 of 3 possible GATA1 binding sites in the human KLF promoter. The other 8 different mutations were found in the KLF1 coding sequence. The effect of these mutations considerably overlapped with those reported in KLF1 null mouse studies (Drissen et al., 2005; Hodge et al., 2006; Pilon et al., 2008). These mutations resulted in individuals with a slightly elevated HbF (1-3%) and their red cells showed gross reduction in expression of Lutheran blood group glycoprotein together with a reduction in Cluster of Differentiation (CD)44 (Singleton et al., 2008).

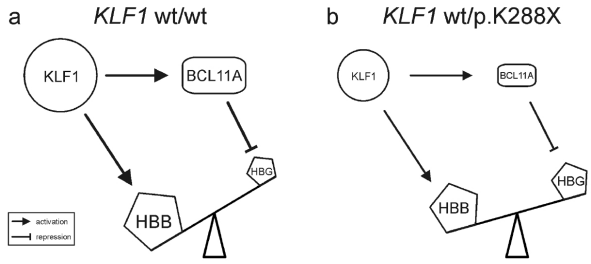

Figure 3: **Model for the regulation of β-like globin expression by KLF1 in adults.** Figure a shows that KLF1 preferentially activates the HBB gene and the BCL11A gene, while the BCL11A protein silences the HBG1/HBG2 (HBG) genes in normal adults. Figure b shows that in patients with KLF1 p.K288X mutation KLF1 activity is reduced. This decreases expression of BCL11A and the diminished amount of BCL11A protein alleviates repression of the HBG1/HBG2 genes. Adapted from Haploinsufficiency for the erythroid transcription factor KLF1 causes Hereditary Persistence of Foetal Haemoglobin, by (Borg et al., 2010).

The role of *in vivo* KLF1 in elevated HbF and control of human haemoglobin switching was shown for the first time in a large Maltese family (Borg et al., 2010). Ten

out of twenty seven family members exhibited Hereditary Persistence of Foetal Haemoglobin (HPFH) with HbF levels ranging from 3.3% to 19.5%. A genome wide linkage analysis on the 27 family members revealed a consistent haplotype at 19p13.12-13 co-segregating with the high HbF. DNA sequencing reveled two linked mutations in KLF1 that were found in all individuals with HPFH. The first mutation was inferred to be a neutral substitution (p.M39L), as mouse KLF1 contains a leucine at this position (Miller and Bieker, 1993). The second mutation is the p.K288X mutation, heterozygous nonsense mutation that involves an alanine (A) to thymine (T) transversion resulting in a lys288-toter (K288X) premature stop codon. This mutation is predicated to ablate the complete zinc finger domain of the protein (Feng et al., 1994), perturbing its function. A random sample of 400 individuals drawn from the general Maltese population did not find KLF1 p.K288X variant. A genome wide expression analysis was carried out on RNA isolated from erythroid progenitors cultured from peripheral blood from four family members with HPFH and four without. These data showed that mild hypochromic microcytic indices shown by individuals with HPFH was due to deregulation of these KLF1 target genes. It was also noted that embryonic *Hbb*-ɣ and HBE1 genes were highly regulated whereas the expression of BCL11A was downregulated in these HPFH individuals. BCL11A is a foetal globin repressor (Sankaran et al., 2008). Downregulation of BCL11A and expression of *HBG1/HBG2* genes was confirmed by quantitative RT-PCR (qPCR). KLF1 knockdown in human erythroid progenitor cells (HEPs) derived from healthy donors was investigated and the quantitative S1 nuclease protection assays showed that knockdown of KLF1 give rise to an increase in *HBG1/HBG2* expression and also a decrease in BLC11A expression both at a protein level and at mRNA level. HEPs were transduced with lentiviral vectors that expressed either the KLF1 p.K288X truncation mutant or full length KLF1. After transduction with full length KLF1, levels of BCL11A protein were increased. This result was not observed after transduction with either Green Fluorescent Protein (GFP) or truncated KLF1 lentiviral vectors. This shows that KLF has a dual role (Figure 3) in the regulation of foetal-to-adult globin gene switching. Primarily it acts directly on the *HBB* locus as a preferential activator of the HBB gene as reported by (Wijgerde et al., 1996) and secondly it acts indirectly by activating the expression of BCL11A which in turns represses the *HBG1/HBG2* genes (Borg et al., 2010; Zhou et al., 2010). In this study it was concluded that haploinsufficiency of KLF1 give rise to HPFH and a fruitful approach in raising HbF levels in individuals with β-type haemoglobinopathies can be achieved by the attenuation of KLF1.

When compared to primitive erythroid progenitors, in definitive erythroid progenitors it was shown that the level of mouse KLF1 increases threefold (Zhou et al., 2006). When carrying out chromatin immunoprecipitation (CHIP) experiments, it was found that this temporal change in KLF1 abundance is due to the differential binding of KLF1 to embryonic/foetal and adult globin gene promoters during development (Zhou et al., 2010). To study the hypothesis that a decrease in KLF1 levels in adult erythroid progenitors will give rise in reactivation of foetal globin gene expression (Zhou et al., 2010) deleted the 50-base pair HS1 enhancer of the mouse KLF1 gene and the mutant allele strain was bred with mice containing a bacterial artificial chromosome carrying a 100-kb insert spanning the human globin locus. It was found that there was an increase in endogenous mouse εɣ2-globin/β-globin and BAC derived human ɣ-globin/β-globin gene expression ratios in livers of embryonic (E) 14.5 animals that were homozygous for the enhancer deletion. It showed that an increase in definitive erythroid progenitors is essential for correct globin gene switching.

The BCL11A RNA and BLC11A protein levels were examined in adult erythroid progenitors from $KLF1^{EHS1\Delta/EHS1\Delta}$ mice and it was found as already concluded by (Borg et al., 2010) that Bcl11a expression is decreased, which in turn augments ɣ-globin gene expression. When carrying out Chip-quantitative PCR (qPCR), (Zhou et al., 2010) found that in adult bone marrow erythroid cells, KLF1 binds to the CACCC box in the mouse BCL11A promoter, whilst (Borg et al., 2010) showed the same in humans. Therefore BCL11A expression is directly regulated by KLF1 *in vivo*.

4 KLF1 mutations and Haemoglobin A_2

One of the most reliable haematological findings for identification of β-thalassaemia carriers is increased haemoglobin A2 (HbA2) between 3.8% to 6.0%. This is not always the case because some atypical carriers have borderline HbA2 levels those between 3.3% - 3.8% (Galanello et al., 1994; Mosca et al., 2008; Giambona et al., 2008). Decreased mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) are usually associated with borderline A2 levels and these are usually the result of mild β+ -thalassaemia mutations, co-inherited δand β-thalassaemia, β-promoter mutations or coexisting iron deficiency anaemia (Harthoorn-Lasthuizen et al., 1999; Galanello et al., 1981). Genetic determinants such as triplication of the α-globin genes, β-promoter mutations and some *HBD* and *HBB* gene variants can give rise to borderline HbA2 with normal MCV and MCH %

(Galanello et al., 1994; Mosca et al., 2008; Giambona et al., 2008). (Perseu et al., 2011) for the first time found that KLF1 gene mutations are responsible for individuals with border line HbA2 and normal MCV and MCH.

In this study 145 subjects with borderline HbA2 (values between 3.3% and 4.1%) and normal or slightly reduced MCV and MCH were studied while 8 normal people were used as controls. The presence of mutations in the *HBB* promoter, triplicated α-globin genes, and haemoglobin variants were excluded. Out of the 145 subjects, 52 had the non-sense KLF1 p.Ser270X mutation, similar to the one reported by (Borg et al., 2010) but slightly more upstream. The p.Thr280_His283del mutation was found in two subjects while the p.Arg319GlufsX34 frameshift mutation was found in four individuals. Two individuals had the p.Thr327Ser mutation while one individual had the p.Lys332Gln missense mutation; another one had the p.Leu326Arg mutation. The p.Ser270X mutation and the Thr280_His283 del mutation lie in exon 2 and 4, while the p.Arg319GlufsX34, p.Leu326Arg, p.Thr327Ser and p.Lys332Gln lie in exon 3. The p.Ser270X and the p.Arg319GlufsX34 mutations result in haploinsufficiency of KLF1 since they ablate the DNA binding domain. On the other hand the p.Thr280_-His283del mutation result in the deletion of cysteine 281 which is crucial for Zn coordination and therefore it eliminates the Zn finger structure and binding to DNA. The other missense mutations that is the p.Leu326Arg, p.Thr327Ser and the p.Lys332Gln mutations affects the amino acids that are adjacent to the residues expected to directly contact DNA. Therefore this might interfere with the binding of KLF1 to DNA. On the other hand these mutations could impair the interaction of KLF1 with Brg1 and Baf156 that were mapped by (Kadam et al., 2000) to the DNA binding domain resulting in the alteration of the chromatin remodeling ability of KLF1.

In the 145 subjects no correlation between HbF levels and known HbF-associated polymorphisms such as the XmnI in the HBG1 gene, rs9399137 in the HBSIL-MYB intergenic region and the rs11886868 in the BCL11A gene was found (Thein et al., 2007; Uda et al., 2008). As previously reported by (Singleton et al., 2008; Borg et al., 2010; Satta et al., 2011) in large series of subjects anaemia was absent in individuals carrying KLF1 mutations. This confirms that one functional KLF1 allele is sufficient to sustain normal human erythropoiesis. Also all the KLF1 mutations were associated with the In(Lu) blood group as reported by (Singleton et al., 2008). This suggests that the amount of KLF1 necessary to regulate Lutheran expression is highly limiting.

5 KLF1 mutations and Foetal Haemoglobin

HPFH is characterized by the presence of elevated foetal haemoglobin in red blood cells of adults. Individuals may be heterozygotes, homozygotes or compound heterozygotes for HPFH (Huisman et al., 1975) and it has been identified in a diverse range of ethnic groups (Giardine et al., 2007). It is well known that HPFH can be caused by deletions within the β-globin gene cluster on chromosome 11 (Henthorn et al., 1990) and point mutations in the promoters of the ɣ-globin genes (Ottolenghi et al., 1989; Wood, 1993). Single nucleotide polymorphisms (SNPs) or oligonuclotide motifs within the β-globin gene cluster are also associated with HPFH. The C-T polymorphism at position -158 of the ^{G}-ɣpromoter which creates an Xmn1 restriction site is the best know of these mutations (Gilman and Huisman, 1985). As already mentioned two major sites that affect the HbF levels that are unlinked to the β-globin gene cluster that have been identified are the HBS1L-MYB intergenic region on chromosome 6q23 (Thein et al., 2007) and the BCL11A on chromosome 2p16.1 (Uda et al., 2008). An addition potential locus associated with HPFH was identified by (Borg et al., 2010) and this was the KLF1 gene.

To examine whether KLF1 mutations are involved in the increased HbF levels a study by (Gallienne et al., 2011) was carried out. In this study a total of 131 samples with elevated HbF levels (between 1.5-25%) together with 121 normal samples with HbF levels $< 1\%$ were tested for KLF1 mutations. Out of 131 patients, 41 were α-thalassaemia carriers, one had Haemoglobin E disease, 6 were carriers for sickle cell trait and 28 were carriers for a β-thalassaemia mutation. In total in these 131 subjects, eleven different KLF1 mutations were identified, nine of which were previously unreported. A polyPhen-2 and SIFT analysis for the mutations in KLF1 identified in 11 out of these 131 subjects predicted to effect gene function. Eight of the eleven mutations were missense mutations. The p.L51R was in exon 1 while p.R301C, R301H, W313C, R328H, R328L, T334K and T334R are in the zinc finger domains. These mutations are expected to disrupt DNA binding. In exon 2, two frame shift mutations were found while an 11 bp deletion, the K54PfxX9, mutation gave rise to new stop codon 8 nucleotides downstream. This mutation is the most severe type of mutation since it gives rise to the loss of all three zinc finger domains and most of exon 2. A G176AfsX179 gave rise to a 7bp insertion producing a stop codon 178 nucleotides downstream. The final mutation identified was a 1 bp nucleotide substitution at the 3' end of exon 2. This mutation is expected to disrupt splicing. In one of the

individuals homozygote for sickle cell mutation a previously unreported KLF1 mutation was identified. This mutation was the c.914-4_-14-1 del CTAG. This individual was completely asymptomatic and maintained a haemoglobin level of 12.7g/dL with HbF level of 20.3%. This shows that the KLF1 mutation is ameliorating the phenotype by increasing the HbF level via reduced ɣ-globin gene suppression.

KLF1 mutations are associated with a spectrum of phenotypes such as the In(Lu)blood group (Singleton et al., 2008), zinc protoporphyria (Satta et al., 2011), Increased HbA2 (Perseu et al., 2011), congenital dyserythropoeitic anaemia (CDA) (Arnaud et al., 2010) and hereditary persistence of foetal haemoglobin (Borg et al., 2010).

6 Kruppel-like factor 10 (KLF10)

KLF10 is situated on chromosome 8q22.2 and functions as a transcriptional repressor involved in the regulation of cell growth. Whole-transcriptome analysis identified molecular signatures of HEP cells isolated from two patient groups representing β-thalassaemia and SCD patients that respond well or do not respond well to Hydroxyurea (HU) treatments, and a healthy adult group whose erythroid progenitor cells were grown with or without addition of HU in culture media (Borg et al., 2011). Looking at molecular signatures in response to HU common probe sets revealed 43 common genes that may be involved in developmental regulation of HbF. These genes can act either directly or indirectly leading to an increase in ɣ-globin synthesis therefore an increase in HbF.

One HU target gene of particular interest that was present in all groups was the KLF10 gene. The KLF10 gene appeared to be significantly associated with high HbF because it appeared in the comparison between low- versus high HbF expressing cells under HU treatment for both the Hellenic β-thalassaemia/SCD compound heterozygotes and the Maltese individuals. It appears that KLF10 is both an HU and a KLF1 target; it appears to influence globin synthesis by acting on genes either directly or indirectly. In the Caucasian populations, within KLF10 four tag SNPs with a minor allele frequency were identified. Two of the SNPs reside in the 3-Untranslated Region (UTR), one is a synonymous-coding SNP in exon 3 and one is intronic. The intronic, SNP namely the rs3191333 polymorphism resides on a regulatory region and therefore it can act as a pharmacogenomic marker by exerting a functional role of KLF10 expression. To see the effect of rs3191333 (c.*141C>T) tag SNP with β-thalassaemia severity (Borg et al., 2011) exploited a large number of β-thalassaemia major patients with low HbF and well-characterized β-thalassaemia intermedia patients with high HbF levels. An independent β-thalassaemia/SCD compound heterozygous patient sample that received HU as therapeutic routine also was included. It was concluded that the absence of the rare homozygous mutant (TT) SNP (rs3191333) is significantly associated with increased HbF in β-thalassaemia intermedia compared with β-thalassaemia major patients and healthy volunteers. In β-thalassaemia intermedia samples the rare allele (T) is less frequent and only present in heterozygous state. Therefore it can be hypothesized that the presence of the T allele in context of β-thalassaemia could be associated with low HbF levels. An inverse correlation in the homozygous normal C/C in responders and homozygous T/T genotypes in non responders was also noted. From the above one can conclude that in homozygous mutant state the rs3131333 SNP renders the KLF10 transcript unstable and therefore the KLF10 gene expression is decreased.

It has been hypothesized that the KLF10 might be acting through SIN3A, especially since the interactions between the two have been already documented by (Zhang et al., 2001). The *SIN3A* is a very important corepressor gene that works together with HDAC1 and physically binds to KLF1 on acetylated lysine residue at position 302 and in turn this represses KLF1 activity. Repressed activity of KLF1 results in decreased adult stage globin synthesis which in turn facilitates the synthesis of foetal globin (Siatecka and Bieker, 2011). It can be hypothesized that though the interaction with *SIN3A*, KLF10 represses the adult HBB gene leading to a higher HBF level (Borg et al., 2011).

7 Kruppel-like factor 2 (KLF2)

KLF2 is a positive regulator of the mouse and human embryonic β-globin genes (Basu et al., 2005). KLF2 was known as lung KLF or LKLF and plays an important role in T-cell differentiation and blood vessel development (McConnell and Yang, 2010). Within their DNA binding domains, KLF1 and KLF2 have high homology and they reside close to each other on chromosome 19 in human and on chromosome 8 in mouse (Basu et al., 2005). In mouse embryonic β-globin genes, KLF1 and KLF2 can partially compensate for each other. It was found that when both KLF1 and KLF2 are ablated in mice, there is more reduction in Eɣand βh1-globin mRNA than when KLF1 or KLF2 single knockdowns are performed (Basu et al., 2007).

(Alhashem et al., 2011) determined whether KLF1 and KLF2 control the human embryonic and foetal β-globin genes and the mechanistic roles of these two genes in globin gene regulation. For this study a mouse model that has the KLF1 and KLF2 knock

out (KO) alleles on the same DNA homology was generated together with transgenic (Tg) mice that carry the entire human β-globin locus. The Tg-HBB mice were bred with KLF1$^{+/-}$ or KLF2$^{+/-}$ mice and therefore KLF1$^{+/-}$Tg-HBB KLF2$^{+/-}$Tg-HBB and KLF1$^{+/-}$KLF2$^{+/-}$Tg-HBB mice were obtained. The mouse embryonic yolk sacs and blood cells were collected followed by RNA and cDNA synthesis and ChIP assays. By comparing the amounts of KLF1 and KLF2 mRNA it was found that the quantities of KLF1 equalled the KLF2 mRNA at E9.5 but KLF1 mRNA increases dramatically by E12.5 whereas KLF2 mRNA levels remain relatively unchanged between E9.5 and E12.5. This shows that at E9.5 both KLF1 and KLF2 regulate the mouse Eγand βh1-globin genes but at E12.5 only KLF1 regulates adult β-globin gene expression.

It was already known by (Basu et al., 2007) that KLF1 and KLF2 regulate the mouse embryonic Eγand βh1 during primitive erythropoiesis, (Alhashem et al., 2011) wanted to test whether these two genes also regulate human β-globin gene expression in embryo. In transgenic mouse models, the human embryonic and foetal β-globin genes, the εand γare both expressed at E10.5 (Jiang et al., 2008). To test this hypothesis dual Tg-HBB and heterozygous KO mice were crossed with heterozygous KO mice to obtain E10.5 KLF1-/-Tg-HBB, KLF2-/-Tg-HBB and KLF1-/-KLF2-/-Tg-HBB embryos. The amounts of human ε- and γ- globin mRNA was measured using qRT-PCR. It was found that ε-globin mRNA was significantly reduced to 15 to 49% in KLF1-/-Tg-HBB and KLF2-/-Tg-HBB yolk sacs when compared with Tg-HBB. On the hand γ-globin mRNA was reduced to 31% of Tg-HBB in KLF1-/-Tg-HBB yolk sacs. In KLF2-/-Tg-HBB yolk sacs the γ-globin gene expression is reduced to 73% showing that KLF2 has a more modest effect on γ-globin mRNA as already discussed by (Basu et al., 2005). When looking at these results one can say that KLF1 appears to have a greater effect on εand γglobin gene expression but with KLF2 also plays an important role. The synergistic regulation of the human εand γglobin genes by KLF1 and KLF2 in transgenic mice models cannot be ruled out.

ChiP assays were carried out to better understand the mechanism by which KLF1 and KLF2 control human and mouse embryonic β-globin gene expression. It was found that at E10.5, KLF1was significantly enriched at the promoters of the Eγand βh1-globin genes at mouse 5'HS2 in the LCR. KLF1 was significantly enriched at the promoter of the γ-globin gene, 5'HS2 and 5'HS3 in human β-globin loci. The pattern of KLF1 enrichment at the mouse and human β-globin loci at E11.5 was similar as that at E10.5, the only difference being that the binding of KLF1 to ε-globin was also seen at E11.5. KLF2 in contrast to KLF1 was detected at the Eγbut not at the βh1-globin promoter at E11.5. This is consistent with (Strouboulis et al., 1992) that at E11.5 there is higher expression of the Eγthan βh1-globin gene. In the mouse β-globin LCR there was no evidence that KLF2 binds to 5'HS2 and 5'HS3 but in human β-globin locus KLF2 was enhanced by about 2-fold at the γ-globin promoter and at 5'HS2 and 5HS3. These results by (Alhashem et al., 2011) show that by direct binding to the CACCC elements in the promoters and LCR KLF1 and KLF2 regulates the embryonic and foetal β-globin genes. As in adult erythroid cells, binding of KLF1 and KLF2 to LCR could be necessary for direct contact between the LCR and the β-globin gene promoter (Drissen et al., 2004).

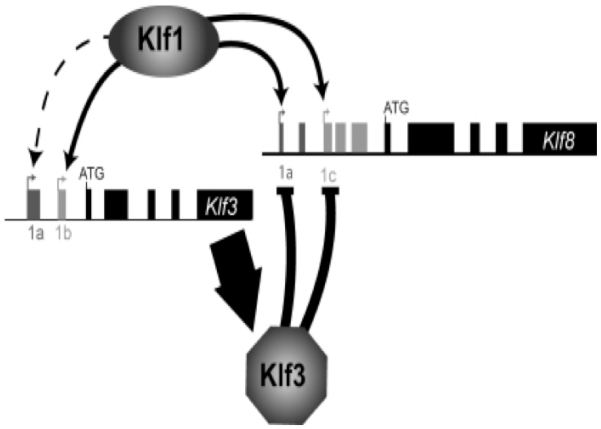

Figure 4: **Schematic diagram of cross-regulation between Klf1, Klf3 and Klf8.** Klf1 activates expression of the Klf3 and Klf8 gene promoters. Klf3 can also repress the Klf8 promoters. In tissues where both Klf1 and Klf3 are present, such as foetal liver, the proteins compete for binding, and this restricts Klf8 to low levels of expression. In the absence of Klf3, for example in the Klf3 null foetal liver, Klf1 gains access to the Klf8 promoters, resulting in de-repression of the gene and increased Klf8 protein (Adapted from (Eaton et al., 2008)).

8 Other Kruppel-like factors

Although some KLFs such as KLF1 primarily function as activators of transcription (Miller and Bieker, 1993), other KLFs such as KLF3 have been described as transcriptional repressors (Turner and Crossley, 1998). On the other hand KLF8 can act as either activator or repressor depending on the biological context and the gene regulatory region through which it is operating (van Vilet et al., 2000; Wei et al., 2006; Urvalek et al., 2010; Urvalek et al., 2011). It was documented (Funnell et al., 2007; Eaton et al., 2008) by that the transcription of KLF3 and KLF8 is carried out by KLF1 as showed in figure 4.

KLF3 that was previously known as basic Kruppel-like factor (BKLF) silences gene expression by the recruitment of co-repressor C-terminal binding protein (CtBP) (Turner and Crossley, 1998; Pearson et al., 2011). Although KLF3 is expressed widely, since it has an ery-

throid specific promoter that is directly activated by KLF1, it is abundant in erythroid tissue (Tallack et al., 2010; Funnell et al., 2007). *In vitro*, KLF3 exhibits similar DNA-binding preferences to KLF1 and in erythroid cells *in vivo* it is as though many genes that are activated by KLF1 are repressed by KLF3 to fine-tune their expression during erythropoiesis (Funnell et al., 2012). KLF8 is one such gene that is activated by KLF1 and repressed by KLF3 (Eaton et al., 2008). KLF3 and KLF8 proteins share 96% of their sequence in their zinc finger domain (Lomberk and Urrutia, 2005) and both proteins recruit CtBP corepressor to silence gene expression (Turner and Crossley, 1998).

The erythroid roles of KLF1 and KLF3 were already investigated but little was known about the contribution of KLF8 to this regulatory network. Therefore Alister et al., (2013) investigated the erythroid role of KLF8. For this study KLF3-/- were crossed with mice with homozygous disruption of KLF8 ($KLF8^{gt/gt}$) followed by histological examination of mice, quantitative real time RT-PCR for KLF8, ε-globin, βh1-globin, ζ-globin and α-globin. Protein overexpression in COS cells was also performed followed by Western blotting, genotyping, ChIP analysis and microarrays. It was found by (Lahiri and Zhao, 2012) that KLF8 protein is normally expressed at very low levels in non-cancerous tissue, Alister et al., (2013) noted that endogenous KLF8 was also seen in wildtype foetal brain and placental tissue.

It was noted that mice deficient in KLF3 were also viable, but KLF3-/-$KLF8^{gt/gt}$ double mutant died at around E14.5. This shows that *in vivo* KLF3 and KLF8 have overlapping roles and they partially compensate in each other's absence. When KLF3-/- mice were analyzed, KLF8 expression in several tissues was upregulated but this was also noted also in erythroid tissue. Alister et al., (2013) hypothesized that KLF3 and KLF8 operate in a regulatory network to control gene expression since it is known that both of them are activated by KLF1 (Eaton et al., 2008). This was confirmed by microarray analysis of $Ter119^{+}$ foetal liver cells from single mutant and double mutant embryos. In the absence of KLF3, 64 genes were unregulated and these were not significantly elevated in KLF3-/-$KLF8^{gt/gt}$ embryos.

By qRT-PCR Alister et al., (2013) confirmed that in KLF3-/- and KLF3-/-$KLF8^{gt/gt}$ embryonic gene expression is derepressed but adult globin expression is unchanged. This can be due to KLF3, which is the primary repressor of embryonic globin expression and in its absence KLF8 is able to partially compensate. Alister et al., (2013) suggest that together KLF3 and KLF8 participate in the silencing of embryonic globins with other repressors such as SOX6, GATA1, YY1, COUP-TF and DRED (Stamatoyannopoulous, 2005; Tanabe et al., 2002; Filipe et al., 1999; Tanimoto et al., 2000). In $Klf3^{-/-}$ and $KLF3^{-/-}KLF8^{gt/gt}$, BCL11A expression was not found to be unchanged when compared to wildtype. This implies that the repression of embryonic globin genes by KLF3 and KLF8 is not indirectly achieved by altering the transcription of these known regulators in erythroid cells (Alister et al., 2013). In $Klf3^{-/-}$ and $KLF3^{-/-}KLF8^{gt/gt}$ it was also noted that in addition to upregulation of the β-like embryonic globins there was also unpregulation of Hba-x expression. Both KLF3 and KLF8 are a pair of transcription regulators that operate in an erythroid transcriptional network downstream of KLF1. The dominant role in regulating gene expression is carried out by KLF3 while KLF8 is able to partially compensate at some loci.

9 Conclusion

As seen in this review, KLFs are a family of DNA-binding transcriptional regulators that are involved in a wide range of biological processes. KLF1, KLF10, KLF2, KLF3 and KLF8 are involved in haemoglobin control. Trying to understand the complex network between these transcription factors and haemoglobin switching can be a fruitful approach to raise therapeutic HbF levels in individuals with β-type haemoglobinopathies treating the underlying dyserythropoiesis and associated complications.

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Research Note

Performance evaluation of Wied Dalam (WDD) seismic station in Malta

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Abstract. The continual operation of a permanent seismograph, now exceeding a couple of decades in some cases, naturally involves changes of hardware and software over time. Nonetheless, the long-term, consistent performance of the seismic station, and the good quality of its data, is very important for national seismic studies investigating the local seismicity, and also important for the international seismological community researching regional tectonics and deep Earth structures. Here we investigate the data availability and quality of the currently only seismic station on Malta (WDD) since its installation in 1995, and establish spectral patterns in the seismic data that may be influenced by diurnal variations, seasonal weather changes, and/or site-specific settings. The results are important for the future deployment of permanent seismic stations on the Maltese islands, and for the analysis of local seismic hazard and ground motion studies.

Keywords WDD station, quality control, seismic ambient noise, power spectral density, probability density function, site effect.

1 Introduction

The deployment of permanent seismic stations across the globe has been taking place for over a century. Seismographs have witnessed numerous changes in instrumentation, data recording and storage, as well as in data communication. Despite the rapid advancement in technology, cheaper equipment, and quicker installations, the proper installation of a seismic station with a good site selection and ongoing station maintenance remains critical for the long-term quality of the seismic data. Moreover, the usefulness of seismic data is greatly increased when 'background' seismic noise levels are low (McNamara and Buland, 2004). Such data is often used for seismic studies relating to regional and global seismicity and investigations of seismic sources and deep earth structures. Here we investigate the overall performance of the permanent station on the island of Malta, Central Mediterranean.

The University of Malta has operated various seismographs since the beginning of the 20th century, when seismographic instrumentation was in its early stages. A Milne-Shaw horizontal pendulum seismograph operated from around 1900 to the 1950's. In 1977 a vertical long-period Sprengnether seismograph with photographic recording was installed. This was replaced some years later by a three-component short period station with analogue paper recording. The seismograms from these instruments are still preserved by the Seismic Monitoring and Research Unit (SMRU) within the Physics Department at the University of Malta. At present the SMRU operates one permanent, broadband seismic station in Wied Dalam (WDD) in the southern part of the island, housed in a disused tunnel at a distance of about 900 metres from the coast (Figure 1). WDD seismic station is located on Lower Coralline limestone, the oldest of the four main geological formations outcropping on the Maltese archipelago. The geology of the Maltese islands is relatively young, with the oldest rock dating back only to the Tertiary period (Pedley et al., 1978; Mourik et al., 2011). The station was installed in June 1995, as part of the MedNet network,

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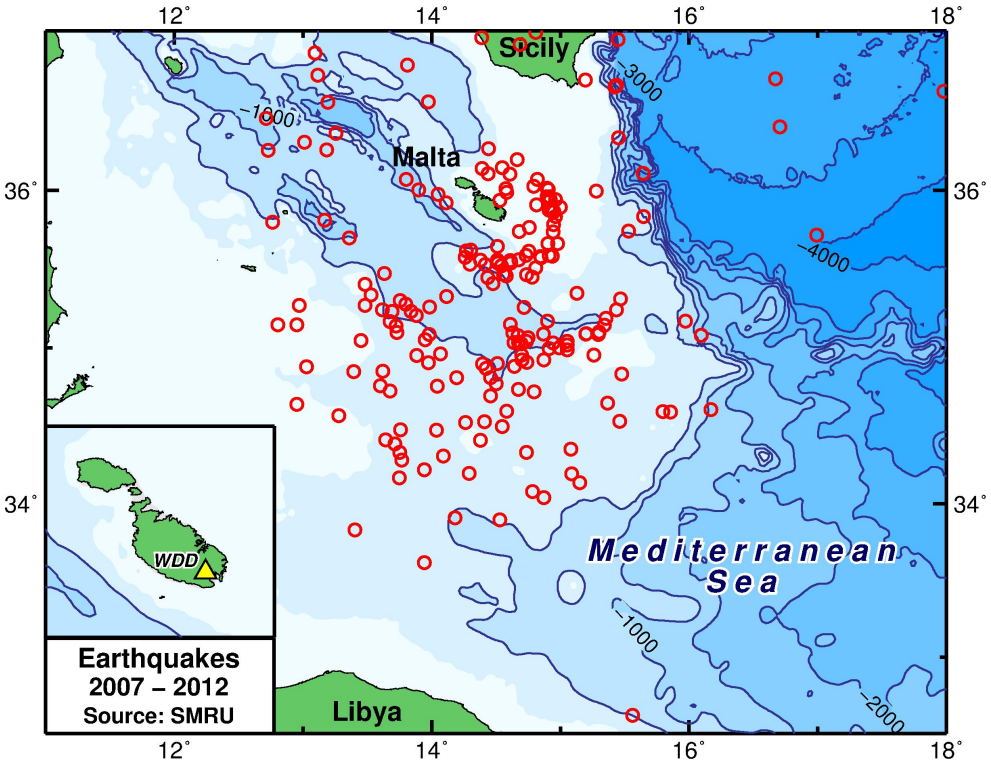

Figure 1: The seismicity around the Maltese islands. Red open circles are epicentres of earthquakes located for the time period 2007–2012. Data is from the online catalogue of the Seismic Monitoring and Research Unit, University of Malta http://seismic.research.um.edu.mt. Different shades of blue indicate the bathymetery in the region. Contour lines show specific depths in metres. Inset shows the location of the permanent broadband seismic station WDD on Malta (yellow triangle)

Figure 2: WDD seismic station. Left: The Quanterra Q680 data acquisition system. Right: The Streckeisen triaxial seismometer placed on a concrete platform.

managed by the Istituto Nazionale di Geofisica e Vulcanologia (INGV) in Rome (Boschi and Morelli, 1994). The present instrumentation consists of a Streckeisen triaxial seismometer (STS-2) and a Quanterra Q680 data acquisition system (Figure 2) that transmits data in real-time to the SMRU at the Physics Department, via the SeedLink Internet protocol. This also enables real-time transmission to several European data centres. WDD also forms part of the Virtual European Broadband Seismic Network (VEBSN) managed by ORFEUS Data Centre in the Netherlands (van Eck et al., 2004).

The Maltese islands have been affected by a number of earthquakes in the historical past, the epicentre of these earthquakes being in the Sicily Channel (bordered by

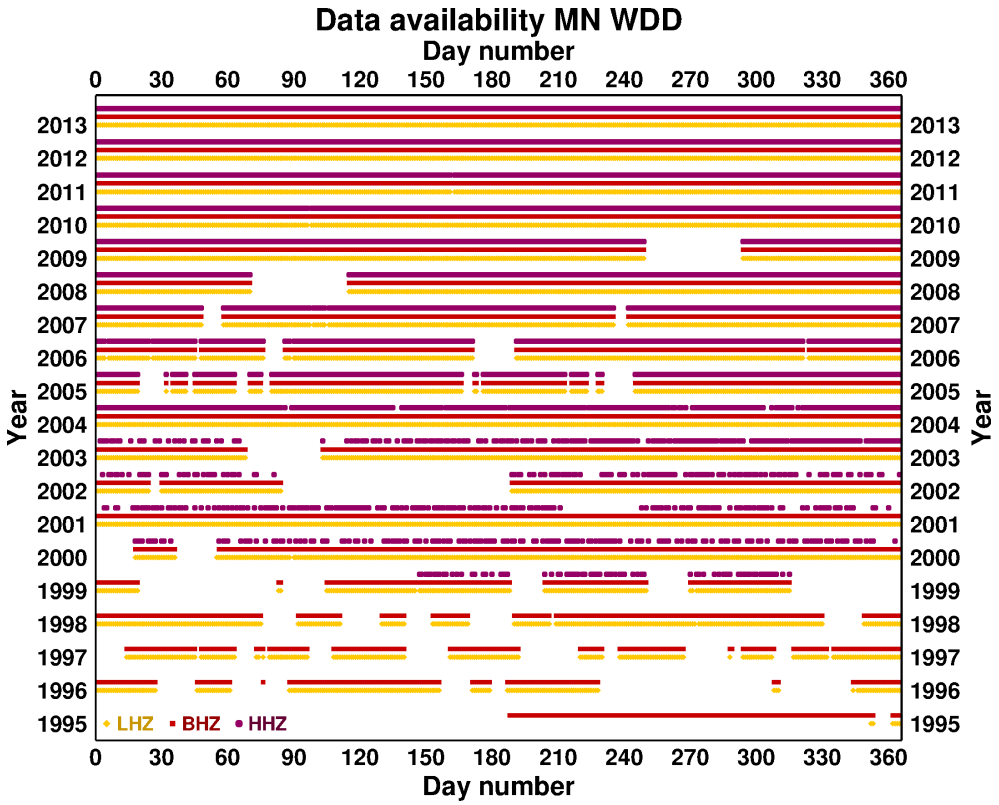

Figure 3: Data availability of station WDD between 1995 and 2013. Each mark represents a data day file of the respective vertical component: LHZ, BHZ, and HHZ.

the Sicilian, Tunisian, and Libyan coastlines), in eastern Sicily, and as far away as the Hellenic arc. Some of these earthquakes produced considerable damage to buildings (Galea, 2007). Since the station's installation, a significant database of earthquakes that have occurred close to the Maltese islands has been compiled. The seismicity for the period 2007–2012 is shown in Figure 1 and is thought to be mainly controlled by active faults in the Sicily Channel rift zone. The earthquakes shown in the figure were located using single-station polarisation analysis (Agius, 2007; Agius and Galea, 2011). This region is characterised by a moderate level of seismicity (Vannucci et al., 2004; D'Amico et al., 2013b) with magnitudes generally below 5. A study on this seismicity is important because it throws light on the activity and seismogenic potential of offshore fault systems. Many of these events, however, are too small to be detected by other European or North African stations, and therefore the presence of a sensitive seismograph on Malta is essential for producing a better picture of the seismicity.

In this study we investigate the data quality of the seismic station WDD and establish spectral patterns in the seismic data that may be influenced by diurnal variations, seasonal weather changes, and/or site-specific settings. The results will be important for the analysis of future installation of permanent seismic stations on the Maltese islands.

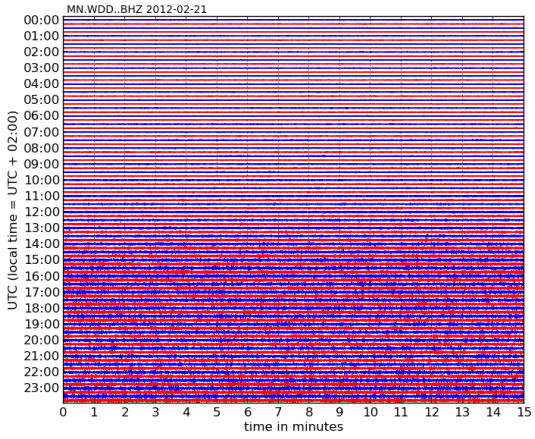

Figure 4: Varying amplitude of microseismic noise. The plot shows 24-hour seismic data of the vertical component (Z) at 20 samples per second (BH) for station WDD on 21st February 2012. Alternating blue and red traces represent 15 minutes of data.

2 Evaluating the seismic data quality

Two very important aspects of running and maintaining a seismic station is the data archiving and the regular analysis of its data quality. Digital seismic data of WDD station is available from international data centres such as ORFEUS and the Incorporated

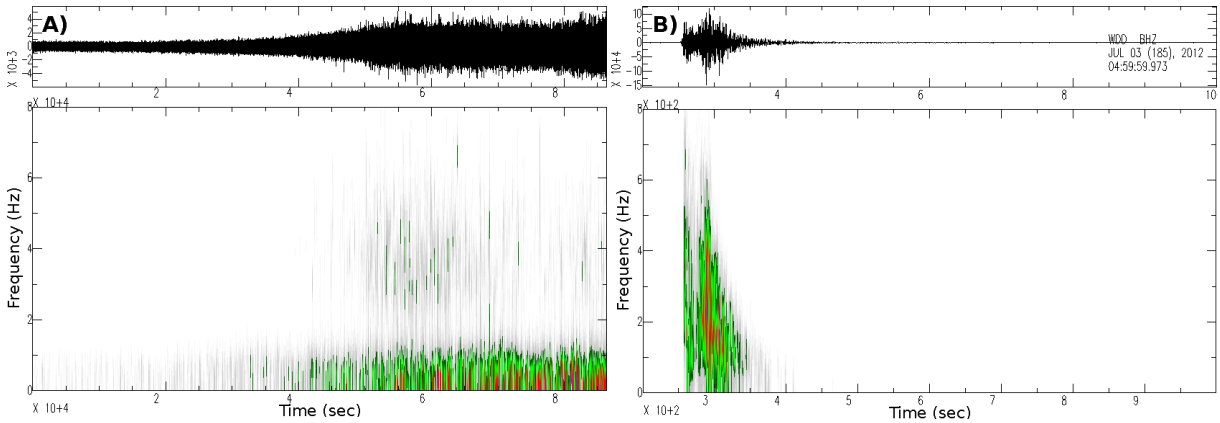

Figure 5: Seismic spectral analysis. A: One day long seismic trace for WDD station, BHZ component, recorded on 21st February 2012 (Figure 4). Below is the spectral analysis showing spectral power as a function of frequency and time. Red spots indicate higher energy. B: A seismic trace of a M 4.8 earthquake, 198 km south-east of Malta recorded at WDD, BHZ component, on 3rd July 2012, and the corresponding spectral analysis.

Research Institutions for Seismology (IRIS); a complete data set (since 1995) is available from the SMRU. The broadband seismic data is available in various channels HH, BH, LH, VH, and UH, with sampling frequency of 80, 20, 1, 0.1, and 0.01 samples per second, respectively. Figure 3 shows the data availability for the the vertical components LHZ, BHZ and HHZ. Continuous seismic data availability has improved over time, uninterrupted for the last years. Gaps in the data can either be from a station shut down due to maintenance, or electric/internet outage. In the earlier years, some loss of HH data is observed because this stream was the first to be erased automatically from the Quanterra hard disk.

Small diurnal variations in the seismic trace are typical and easily noticed visually on a 24-hour data plot such as Figure 4 for the 21st February 2012. The amplitude of the trace increases in the second half of the day. Such changes are usually weather related, as is the case on this particular day. On this date the islands experienced a severe rain storm that resulted in the dullest (lack of sun radiance due to thick clouds) day of the month with heavy rainfall of 24.2 mm ("February was the third coldest in almost a century" The Times, 2nd March 2012, p. 5., print). The increase in amplitude of the seismic trace is due to an increase in microseisms, background seismic noise generated by the storm. Because Malta is a small island, microseismic signals are dominated by the continuous swelling of the surrounding sea crashing onto the shore. These were amplified on the day of the storm.

Another way to inspect seismic data is by analysing the spectral content. Different sources of seismic signals, earthquake or otherwise, have a spectral signature. Figure 5 shows two examples of a spectral analysis, one showing the spectrogram of seismic ambient noise recorded throughout the day of the 21st February 2012 (same as Figure 4), and another spectrogram of a regional earthquake recorded on the 3rd July 2012 capturing a M 4.8 earthquake, 198 km south-east of Malta. The dominant frequencies for the ambient noise are between 0 and 1 Hz and are persistent throughout the day like a permanent hum. In the case of the earthquake signal the dominant frequencies are higher, in the range of 0 to 6 Hz. The seismogram of an earthquake has a different spectral and energetic content for the different arriving phases such as body waves and surface waves; hence the dominant frequencies are within a broad range. Eventually the energy decays with time, back to the background noise.

A powerful tool to evaluate the long-term performance of a broadband seismic station is the overview of the accumulative spectral content over a series of days, weeks, or months (Figure 6). This gives one the ability to examine artefacts related to station operation, episodic cultural noise (for example, day variations due to traffic) and seasonal weather changes — together they can form a baseline level of ambient noise of a certain site. Such an analysis is useful for characterising the current and past performance of a broadband sensor, for detecting operational problems, and for evaluating the overall quality of data of the station.

In this study we use the approach described by McNamara and Boaz (2006), which incorporates a probability density function (PDF) to display the distribution of seismic power spectral density (PSD). Figure 6 shows selected examples of PSD sampling the months of February and August, for the years 1996 and 2012. Each individual PSD curve represents a 30–minute segment of data. Most of the PSD curves follow a similar distribution of power spectra because most of the data contains background seismic noise, whereas the scattered curves

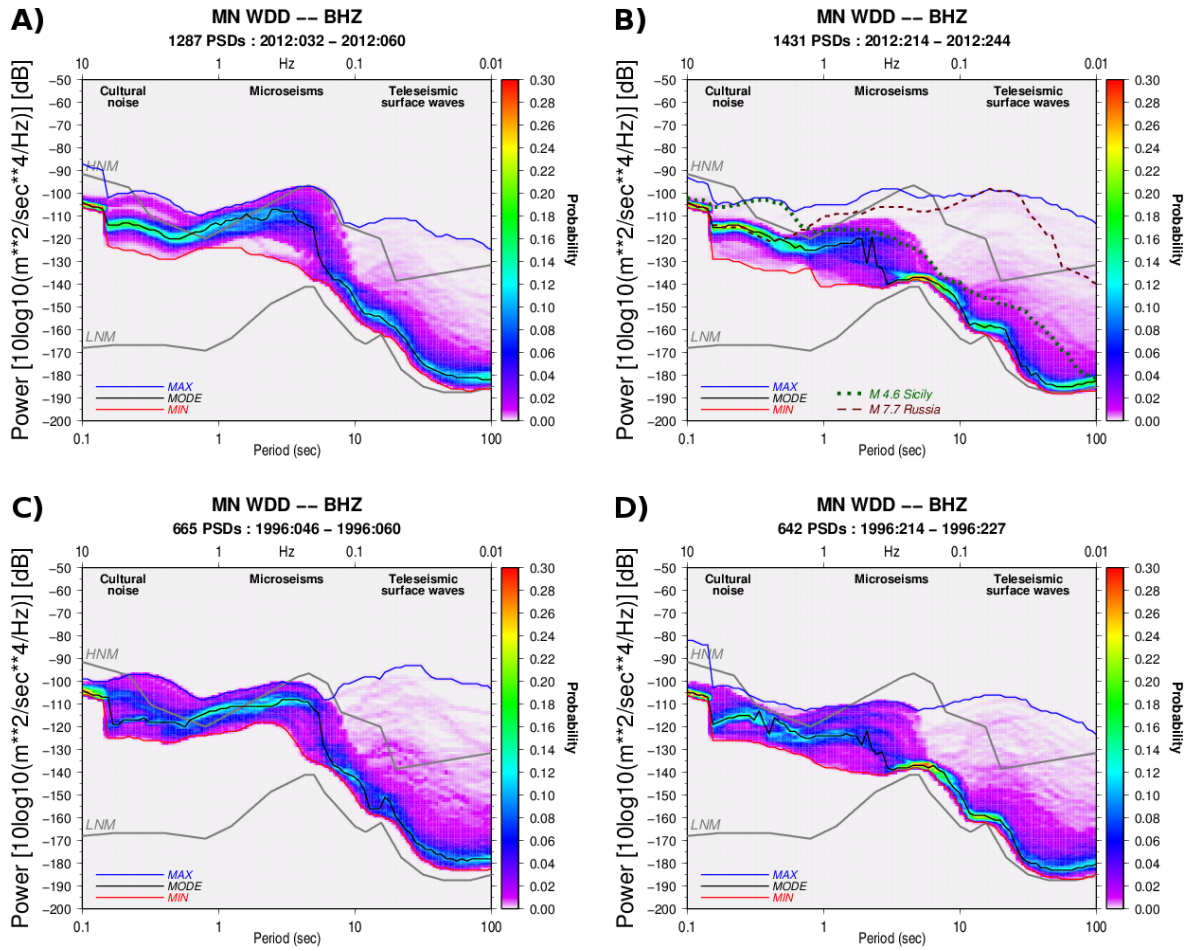

Figure 6: Seismic spectral analysis based on the calculation of Power Spectral Density (PSD) distribution using a Probability Density Function (PDF) (McNamara and Boaz, 2006). A: Noise analysis for the winter period using the seismic data of February 2012, day numbers 032–060. Grey curves: low noise model (LNM) and high noise model (HNM) (Peterson, 1993). Blue and red curves: maximum and minimum PSD for the data, respectively. Black curve: the highest probability mode. B: same as A, but for the summer period using the seismic data of August 2012, day numbers 214–244. Red, thin, dashed curve: the PSD for a teleseismic earthquake that occurred in Sea of Okhotsk, Russia on August 14th with a magnitude 7.7. Green, thick, dashed curve: the PSD for a regional earthquake that occurred in Sicily, Italy on August 28th with a magnitude 4.6. C and D: Same as A and B but for 1996. Less PSD curves are due to less data availability (Figure 3)

are of sparse earthquakes that occur over time. In Figure 6B, August 2012 plot, we show PSD examples of two earthquakes, a teleseismic and a regional event. The spectral content of the teleseismic event is dominant for periods longer than 1 second up to and exceeding 100 s, whereas for the regional earthquake the dominant spectra is for periods less than 1 second (> 1 Hz).

Ideally, background noise is of a low power order throughout the entire spectrum, but this is hardly ever the case; stations such as those close to the coast have an increased power level, particularly for short period waves (less than 10 seconds). Peterson (1993) has used the seismic data of a world-wide network of seismographs to establish a low noise model (LNM) and a high noise model (HNM) shown as grey curves in Figure 6. These models are used for comparison and serve as an indication of how a seismic station performs in terms of ambient seismic noise. During the month of February of 1996 and 2012, WDD has high power levels for periods between 0.2 and 10 seconds (0.1–5 Hz) (Figure 6 A and C). Although the highest probability mode only exceeds the HNM for periods close to 1 second (1 Hz), the broad range of high probability spectral distribution is considered excessive.

During the month of August (Figure 6 B and D), the power spectral distributions for the periods between 1 and 10 seconds (0.1–1 Hz) decrease substantially and are well within the low and high noise models. The amplitude contrast between February and August within the 1–10 second period band is assumed to be due to seasonal changes; very short-period spectra from cultural noise and long-period spectra from teleseismic events have a similar distribution in both months and are unaffected by weather changes. Note that the PSD for the

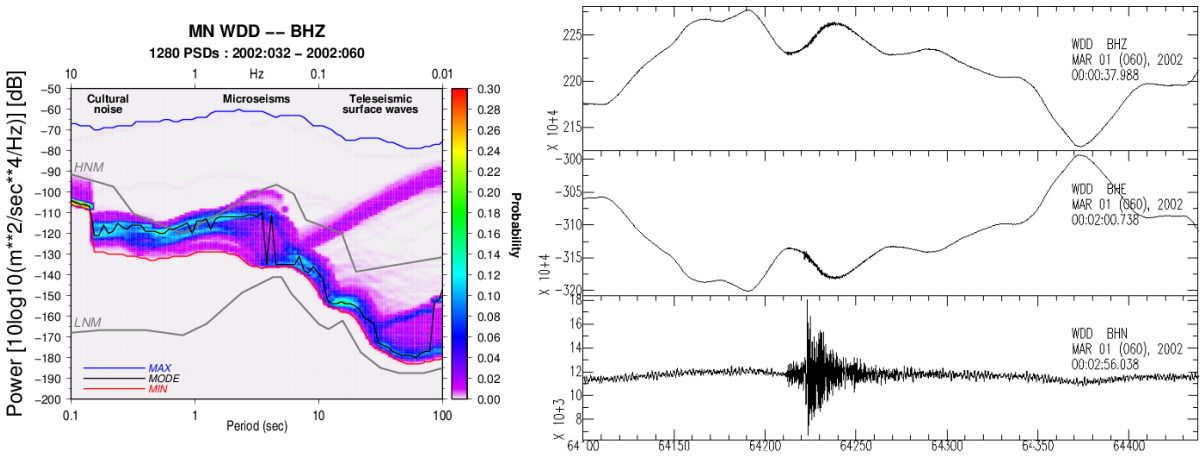

Figure 7: Example of poor seismic data quality. Left: The power spectral density distribution (McNamara and Boaz, 2006) for the BHZ component data for day numbers 032–060 of year 2002. Right: A BH, 3-component seismic trace of a local earthquake recorded during the same time period.

months of 1996 have less samples due to gaps in the data. Nevertheless, the very similar spectral patterns observed for the same months of 1996 and 2012 indicates that the conditions of the seismic station remained the same over the years.

3 Example of poor data quality

Figure 7 shows an example of poor seismic data quality in early 2002. Abnormal spectral patterns can be seen at the longer periods of the PSD for the BHZ component. The long period artefacts are also noticeable in the seismic trace of a local earthquake (Figure 7B). Components BHZ and BHE have a suspiciously long period, large amplitude signal inverted to one another whereas component BHN seems to be unaffected. Although the long period signal can be filtered out to 'save' the data for further processing such patterns are indicative of station operational problems. In such cases maintenance typically requires an on-site inspection, checking the instrument state-of-health for system warnings, and mass re-centering.

4 Station orientation

Various seismic studies, such as shear-wave splitting, depend on the station orientation, generally assumed to be correct (Ringler et al., 2013). Typically the station orientation is done manually during deployment by orienting the north-south station axes to point to the true north using a standard compass. One way to confirm the station's orientation is by locating earthquakes from three component (E, N, and Z) polarisation analysis and comparing the location with that published by international bulletins. This technique has been used by the SMRU to locate as many earthquakes in the Mediterranean region, particularly those occurring in southern Italy and Greece (Agius, 2007; Agius and Galea, 2011). The successful location of earthquakes, particularly those with a large signal-to-noise ratio, gives us confidence that the station orientation is correct, relative to the true north.

5 Seismic site response using seismic ambient noise and earthquake recordings

It is well established that earthquake ground shaking is not only a function of the earthquake magnitude and epicentral distance, but also of the site conditions. Seismic waves propagating close to the surface are strongly affected by the underlying near-surface structures such as soft layers in the sub-soil stratification, and by topographical features. Several studies show numerous examples of anomalous shaking amplification as a result of site effects (Borcherdt and Glassmoyer, 1994; Higashi and Sasatani, 2000; Aguirre and Irikura, 1997; Fukushima et al., 2000; Akinci et al., 2010; D'Amico et al., 2010; D'Amico et al., 2013a). Evaluating the site response of station WDD from local effects will help establish whether the PSD patterns for periods < 2 seconds (Figure 6) are site dependent. Furthermore, the spectral patterns of the site response can help in the site selection of future installations of permanent seismic stations on the islands, and can also be used for an accurate and proper calibration of seismic hazard evaluation and regional ground motion studies (D'Amico et al., 2012a; D'Amico et al., 2012b; Akinci et al., 2013).

In order to determine the site response of station WDD we select 16 local seismic events that have a good signal-to-noise ratio. The magnitude of the events are in the range of 2.5 to 4.3, with a travel path in the range of a few kilometres to about 100 km, and with a back-azimuth ranging between 30° and 250°. Data

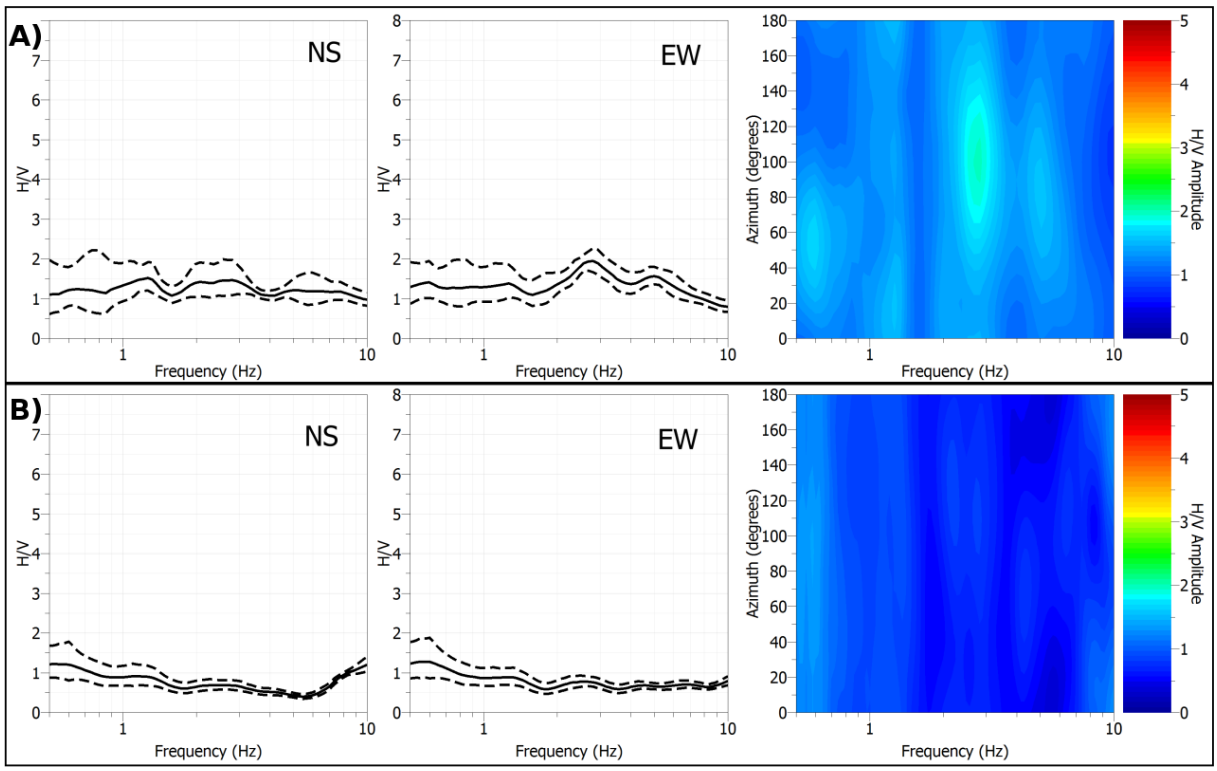

Figure 8: Horizontal to vertical spectra ratio using earthquake and noise recordings. A: HVSR obtained using earthquake recordings at WDD station. Left: Ratio of the north-south horizontal component with the vertical component (solid line). Dashed lines represent the confidence interval. Centre: Same as left, but ratio is of the east-west horizontal component with the vertical component. Right: H/V spectral energy with respect to frequency and azimuth (from north). B: same as A but for HVNSR obtained using noise recordings

were processed for horizontal to vertical spectra ratio (HVSR, Nakamura (1989)). The signals of the recorded earthquakes were base-line corrected, with the purpose of removing spurious offsets, and band-pass filtered in the range 0.08–20 Hz using a fourth order causal Butterworth filter. The analysis was performed considering time windows of 20 seconds, starting from S waves onset and using a 5% cosine-tapered window. The obtained Fourier spectra for each considered earthquake were smoothed using a proportional 20% triangular window. For each window the spectral ratio as a function of the frequency was computed, then the geometric mean was calculated We also performed analysis on a 40 minute ambient noise recording of station WDD. The data was processed using the horizontal to vertical noise spectral ratio (HVNSR) technique following the criteria suggested by SESAME project (Bard, 2004). The Fourier spectra were calculated in the frequency range 0.5–10.0 Hz and smoothed using a proportional 20% triangular window.

Figure 8 shows the obtained HVSR and HVNSR results using the north-south components and the east-west horizontal components separately. In both cases the H/V ratios at the site have a relatively flat behaviour and they do not show any significant peak. According to the SESAME (Bard, 2004) guidelines only the spectral ratio peaks having amplitude greater than two units can be considered significant. Therefore we can state that the WDD station has a flat rock response and it does not present any significant site effect due to the local geology. In Figure 8, the directional analysis is also presented. The amplitude is plotted with respect to azimuth and frequency for the averaged components. The noise analysis shows no directivity at all, whereas the earthquake data shows slight clustering at about 3 Hz signal around N100°E. No clear explanation can be given at this stage and further analysis on a larger data set is needed in order to explain this behaviour.

The flat H/V response of station WDD is attributed to the underlying Lower Coralline Limestone bedrock, the oldest of the geological formations outcropping on the Maltese archipelago. Other similar studies for areas in the northern parts of the islands that have outcrops of a younger geological formation show a different spectral pattern; the site response from temporary stations have a H/V peak amplitude within the frequency range of 1 and 5 Hz (Vella et al., 2013). Hence, station WDD and the site location itself can be used as a local reference, where the seismic data, unlike in other areas on the islands, is 'unaffected' by the underlying geology.

6 Conclusion

We conclude that WDD seismic station has a good performance history. The station has long-term (since 1995), broad-band seismic data availability. Spectral analysis of the data shows that the seismic power spectral density is within the global standard low and high noise model ranges (Peterson, 1993) for most frequencies. Noise levels are high for the frequency ranges of 0.1–5 Hz only during the winter months, due to weather storms. Seasonal variations in noise amplitude are consistent throughout the station's operational years and suggest a stable performance since installation. Analysis on the ambient seismic noise and earthquake data show WDD has a flat site response, indicating minimal effect from the station location. The stable geology at Wied Dalam and the high-quality, broad-band seismic instrument at WDD seismograph can serve as a local reference for calibration of future deployment of permanent seismic stations, as well as a reference for other seismic studies such as site spectral ratio techniques, seismic hazard evaluation and local ground motion studies of the Maltese islands.

Acknowledgments

Some figures were created using the Generic Mapping Tools (Wessel and Smith, 1998), Python toolbox for seismologists (Beyreuther et al., 2010), and SAC2000 (Goldstein et al., 2003). This study was supported by SIMIT Project part-financed by the European Union, European Regional Development Fund (ERDF) under the Italia-Malta Cross-Border Cooperation Programme, 2007-2013.

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Review Article

The male to female ratio at birth

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Abstract. The factors that influence the male to female ratio at birth are legion. Males are usually born in excess and stress decreases the ratio while wellbeing and good health tends to increase it. This paper reviews the multitudes of factors that have been implicated as affecting this ratio, from historical times to date.

Keywords Sex Ratio - Birth Rate/*trends - Infant, Newborn.

1 The male to female ratio at birth

> "The examination of sex distribution in human society constitutes a rather technical, if not abstruse investigation better left to demographers"

(Attané and Guilmoto, 2007).

2 Historical aspects

The male to female ratio of live births is generally expressed as the ratio of male live births divided by total live births (M/F). Although this would be more accurately abbreviated as M/T (male births divided by total births), it is widely (albeit technically incorrectly) abbreviated as M/F, and this will be used throughout.

In ancient times, it was widely believed that an infant's gender was determined by the degree of heat that a man's ejaculate was exposed to during insemination.

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A statistical study of M/F requires not only raw data but also statistical tools for calculations that provide probabilities of deviation from preset values. The collection of the data from London in the 1600s allowed John Graunt to publish the first descriptive statistical analysis of M/F data (Graunt, 1899).

Graunt's publication included a detailed description and analysis of annual variation in M/F in London and Romsey. Graunt noted that "that there be more males than females" and that "London is somewhat more apt to produce males than the country". He based this on the observation that during 1629-1661, 139,782 males and 130,866 females were christened in London, which he approximated as 14:13, a M/F of 0.5164. However, in Romsey, during 1569-1658, 3,256 males and 3,083 females were christened (16:15), a M/F of 0.5136. This is statistically non-significant and Graunt simply speculated that there may be geographic variations in M/F. He also noted secular variation in M/F which is significant with modern day testing.

John Arbuthnott (1667-1735) was a mathematics teacher in London who went on to study medicine (Campbell, 2001). He suggested that "provident Nature, by the Disposal of its wise Creator, brings forth more Males than Females, and that in almost a constant proportion", noting that male excess is God's method for compensating for increased male mortality since "polygamy is contrary to the Law of Nature and Justice".

Arbuthnott's originality lay in his demonstration that M/F is significantly in excess of 0.5 and this is considered the first use of inferential statistics. His calculations compared annual male and female births in London with the outcome of a number of throws with a two-sided die, a sign test (Campbell, 2001).

The Fisherian explanation is that were male births less common than female, a male would have better mating

prospects and would sire more offspring. Thus, parents genetically disposed to produce males would have more offspring and this tendency would spread within the community, increasing male births, such that this advantage disappears when an M/F of 0.5 is reached. The converse would apply were there a dearth of females. Hence "it follows that the sex ratio will so adjust itself, under the influence of Natural Selection, that the total parental expenditure incurred in respect of children of each sex, shall be equal" (Fisher, 1930).

While this theory was popularised by Fisher, it must be pointed out that it had already been mathematically expounded by Carl Düsing of the University of Jena in three publications in 1883 and 1884. These investigations comprised the first application of mathematical methods and models to evolutionary biology (Edwards, 1960).

3 Broad epidemiological aspects of human M/F

Random meiosis would lead to a mean (Mendelian) M/F of 0.5, with binomial variation around this value. However, this is based on the following:

1. Males produce equal numbers of X- and Y-bearing sperm in mammalian species.

2. X- and Y-bearing sperm stand equal chances of achieving conception.

3. Equal numbers of male and female zygotes are conceived.

Thus, any M/F variation would be due to sex-selective foetal wastage. However, evidence has been put forward against these conclusions, as will be demonstrated. In humans, M/F exhibits a male excess, and is expected to approximate 0.515 with a range of 0.505 to 0.520 (James, 1997).

The excess of male births may be nature's compensatory mechanism for increased postnatal male mortality. However, the reason/s and mechanism/s whereby this occurs are uncertain, and several theoretical models have been proposed to explain these variations. This is because a remarkable degree of inhomogeneity in M/F has been demonstrated, both secularly and across regions and the following section will review factors that have been implicated as resulting in these disparities (James, 1997; James, 1987b).

4 M/F physiology

For humans, the gender ratio at birth is determined at the time of conception through genetic control. A review has indicated that there is a consistent and significant excess of males at fertilisation (Boklage, 2005). However, there is evidence that M/F is partially influenced by a combination of endogenous and exogenous factors.

Although the mechanism/s that produce this effect could theoretically operate at conception or during pregnancy, the latter seems more likely. Indeed, it has been argued that a there is no plausible mechanism whereby a species with sets of chromosomes (diploid pairs) could alter M/F at conception (Smits and Maynard-Smith, 1978). Furthermore, it has been pointed out that any maternal control over M/F will be actively opposed by sperm, in that individual sperm that allow themselves to be identified as X- or Y-bearing sperm will not be selected as often as sperm that are able to disguise their gender-selecting material (Reiss, 1987).

Recent studies that rely on the biochemical detection of pregnancy reveal that around 73% of natural singleton conceptions fail to survive beyond six weeks of gestation. This implies that pregnancy is an opportunity not only for gestation but also for selection and/or culling, and that significant wastage occurs before maternal or clinical recognition of pregnancy - 90% of the survivors go on to reach term (Boklage, 1989).

It is also believed that multiple pregnancies may constitute over 12% of all natural conceptions, but only approximately two percent reach term as live twin births, and 12% of these result in single births. In all of the above situations, the attrition described can be modelled with a simple equation for exponential decay (Boklage, 1989).

The sex ratio at conception in humans may be 0.545, with the highest sex ratio of fetal deaths in the second trimester, decreasing in the third trimester, and peaking again at term. This data also suggests that late fetal deaths may be postponed to early infancy (McMillen, 1979).

Evolutionary theory proposes that mutations produce some individuals who may be fitter in a given environment and who are therefore likelier to survive and procreate, dispersing their advantageous genes. One such adaptation could be the ability of the individual to influence M/F outcomes in conceptions.

In polygynous species, only the fittest males reproduce. For this reason, parental investment in a "good quality" son may yield greater numbers of descendants than an equivalent investment in a "good quality" daughter. It may therefore be advantageous for a mother to produce sons when she has sufficient resources to give them a better than average edge that will then give them a greater chance to reproduce, and daughters when she does not. This is known as the Trivers-Willard hypothesis (Trivers and Willard, 1973).

5 Factors known to influence M/F

Three mathematical models have been proposed as potential explanations, individually or in combination/s, to elucidate the probability p of a male birth within and across sibships (Edwards, 1960; James, 1997).

Markov variation assumes that p varies within couples depending on the gender of previous births. Markov variation is said to be positive when p increases with previous male births, and vice-versa.

Poisson variation assumes that p varies within couples from one pregnancy to the next, irrespective of the gender of previous siblings, and has the same overall mean across couples. Poisson variation is "chaotic" if p varies in a random manner within couples. It is called "systematic" variability when p varies from one pregnancy to the next in parallel across all mothers, such as, for example, a birth order effect.

This is complicated by a so-called "stopping rule" of which there are two types. Type I occurs when families desire one or more children of one sex, and cease reproducing when their wish is satisfied. Type II occurs when families desire both sexes among their offpsring, and cease reproducing when their wish is satisfied (James, 1997).

Some form of variation probably operates on M/F as it has been shown, for example, that M/F of sibs of male-male, male-female and female-female twin pairs differ significantly, with values of 0.536, 0.523, and 0.508 respectively (Turpin and Schutzenberqer, 1952). Overall, it appears that the evidence for either Lexis and/or Markov variations exist and this will be briefly alluded to in the next sections (James, 1997).

While the physiological basis for the influences of external factors on M/F are not understood, alterations in parental sex hormone level/s and/or differential gender-based survival modulated by stress during embryogenesis have been proposed as likely mediators.

The hormonal theory is heavily subscribed to by William H. James, the foremost expert in the field (James, 2004). Indeed, a hormonal explanation by this same author will be noted for almost all of the factors that are known to influence M/F. More recently, this same authority has also acknowledged the effect of stressors on M/F during pregnancy, as will be outlined.

Population/physiological differences - Lexis variation The male excess has been historically shown to be significantly less in Black populations when compared to Caucasian populations (Ciocco, 1938; Visaria, 1967), and significantly higher in Asian populations (James, 1997; Visaria, 1967). A more recent study in the United States confirmed the Black-Caucasian difference and also demonstrated a low Hispanic M/F (Branum et al., 2009).

This difference persists even when races co-exist such as in South Africa and the West Indies (Visaria, 1967) and in England and Wales (James, 1997). It has been speculated that this may be due to innate minor physiological differences (Ciocco, 1938; Visaria, 1967). With regard to hormones, the evidence to date seems to indicate that mammalian (including human) M/F is causally related to periconceptual parental hormone levels. For example, differences in the levels of maternal gonadotrophins have been implicated, with higher levels causing a lower M/F (James, 1984). In addition, elevated levels of testosterone and oestrogen increase M/F (James, 1986b) while progesterones decrease M/F (James, 2004).

The luteal surge in the middle of the menstrual cycle has therefore been proposed to be the cause of the excess conception of females noted in the fertile part of the cycle (James, 2004). Interestingly, it has been noted that right-sided ovulation is associated with a higher M/F than left-sided ovulation (Schoner, 1927). Furthermore, serum estradiol and testosterone concentrations are higher in right-sided than left-sided ovulation (Fukuda et al., 2000). It has been speculated that the former may be due to the latter (James, 2004).

Physiological changes in normal hormone profiles may also occur with maternal age, such that a shift in maternal age, for example, to older mothers, may also potentially influence M/F (James, 1984). It has been speculated this may be due to the increasing circulating gonadotrophin levels present in older mothers that favour a lower M/F (James, 1980b).

Furthermore, births to younger fathers and births of lower orders appear to be likelier to be male, increasing M/F (Chahnazarian, 1988). This is particularly relevant since family size has significantly decreased in many countries (Festy, 1984).

However, studies between races, such as Scotland for the period 1975-1988 (549048 $1^{st} - 5^{th}$ order births in 330088 women) have failed to demonstrate Lexis variation (Maconochie and Roman, 1997).

Moreover, it has been shown that the M/F of offspring of women who have never had a spontaneous abortion is very close to that of the surviving offspring of women who have had a spontaneous abortion, further arguing against Lexis variation (Boldrini, 1937; Colombo, 1957).

6 Family planning, birth order, polygamy and multiple pregnancy

Birth order has been shown to be negatively correlated with M/F (Garenne, 2008), being highest among first-born children and declining asymptotically with increasing numbers of offspring (Russell, 1936), a form of systematic Poisson variation.

However, gender preference may potentially influence M/F. For example, couples with a male preference may stop trying to have children if the first birth or the first several births are male, a stopping rule (Goodman, 1961). Indeed, it has been shown that M/F declines with increasing birth order. Moreover, M/F decreases with increasing paternal age (James, 1987a), and (to a lesser extent) with increasing maternal age (Ulizzi and Zonta, 1995). The latter is attributed to increasing circulating gonadotrophin levels present in older mothers that favour a lower M/F (James, 1980b). The former has been attributed to decreasing coital rates (James, 1980a) and to declining male androgen levels with increasing paternal age (Punifoy et al., 1981).

Additionally, the decreased M/F seen with increasing birth order and with increasing paternal age are almost identical, and may be caused by the same factor/s (Novitski and Sandler, 1956).

Interestingly, yet another study has not only shown that maternal age correlates negatively with M/F (40-49 years), but also that very young mothers (12-19 years) also have a low M/F (Garenne, 2008).

Matters are complicated by the fact that consistency between studies is not always present. For example, a large study showed a decline in M/F with paternal age and with number of siblings per plural birth, with no influence from maternal age or birth order (Jacobsen et al., 1999).

In addition, coital rates after marriage have been shown to approximately halve in the first year of marriage and halve again over another twenty years (James, 1983). The relation between M/F and coital rates is confirmed by the finding that M/F declines according to month of conception over the first year of marriage (Bernstein, 1958).

Moreover, twin births (Bulmer, 1970) and multiple births have lower M/F than singleton births (Pollard, 1969), and this has been attributed to the increased male mortality in such multiple pregnancies (Zahalkova, 1978). This is complicated by the fact that periconceptual high levels of maternal gonadotropin predispose to dizygotic twinning (Bulmer, 1970).

In addition, the three main races exhibit different dizygotic twinning rates. Blacks have higher rates than caucasians, who in turn, have higher rates than orientals (Bulmer, 1970). There is also evidence that gonadotrophin levels vary consistently across these three races (Milham, 1964), futher influencing M/F.

7 Warfare and time of coitus in menstrual cycle

Marked increases in M/F in the range of 1-2% have been noted during and after warfare in England, Wales and France in relation to the First and Second World Wars (James, 1987a).

WW1 A rise in M/F was noted in Austria, Belgium, Bulgaria, England, France, Germany, Hungary, Italy, Romania, and South Africa (Russell, 1936; Bernstein, 1958).

WW2 In England and Wales, M/F was higher in 1941-1946 than in any years previously recorded, with registrations dating back to 1841 (Lowe and McKeown, 1950). Similar findings were noted for Belgium, France, Germany and the Netherlands (Graffelman and Hoeksrtra, 2000), as well as in Finland (Vartiainen et al., 1999). This effect was smaller or absent in neutral countries (Russell, 1936), and absent in the United States where the percentage of the population in the armed forces at any one time was less than 4%, compared to 15-22% in the principally affected European countries (Anon, 1939).

Warfare related alteration in M/F has been attributed to coital frequency. In times of war, an adult sex ratio imbalance prevails, with more males being away from their homes. This results in sexual excesses, "actions [that] were viewed as understandable responses to the Frauenuberschuss," the excess supply of women (Moeller, 1993). It has been mooted that in wartime, nonprogrammed copulation and high coital rates co-exist, with more conceptions occurring early or late in menstrual cycle, increasing M/F (James, 1980a).

This is due to the fact that M/F follows a U-shaped regression on cycle day of insemination, suggesting that female conceptions result most often from conceptions around ovulation, with male conceptions occurring more frequently at the beginning and end of the menstrual cycle (Guerrero, 1974; Harlap, 1979). These findings have been confirmed by more recent meta-analysis (Gray, 1991).

This U-shaped regression is confirmed by the higher M/F that is depicted after the failure of rhythm methods of birth control since such failures would theoretically, on average, occur earlier or later in the menstrual cycle (James, 1987b).

It has been noted that coital frequency may be related to individual age, and therefore secular changes

in parental age composition could produce M/F shifts. However, these wartime rises could not be entirely accounted for by changes in fetal death rates, maternal age, parity or birth interval (MacMahon and Pugh, 1953).

Conversely, brief episodes of belligerence decrease M/F. For example, the very short war in Slovenia (26 June-7 July, 1991) reduced M/F to 0.504 in Slovenia and to 0.483 in Ljubljana, 6 to 9 months later. A decrease in sperm motility was also noted, from 56% just before the war to 52% after (Zorn et al., 2002), and this will be further amplified later.

8 Stress and socioeconomic status

This hypothesis implies that natural selection has developed mechanisms by which pregnant females subjected to environmental stressors manipulate M/F by culling male fetuses that are least likely to eventually sire grandchildren. Males are specifically selected for abortion as a male in poor condition is likelier to die before reaching reproductive age than a female in similar condition, despite receiving a greater maternal investment - a socioeconomic paradigm. Mammalian demographic studies support this theory since, as the maternal condition deteriorates, females produce less male offspring (Trivers and Willard, 1973). However, overall, the evidence for this hypothesis is mixed. Reviews have shown that 89 tests of the hypothesis on primates (including man) have only supported it half of the time. Furthermore, some test results went contrary to this hypothesis (Brown and Silk, 2002; Lazarus, 2002).

Male vulnerability is manifest in premature births, as well as in term babies, with higher morbidity and mortality rates that persevere into early childhood. It has therefore been suggested that postnatally, malnutrition, interacting with infection, is a precipitant for male loss. Furthermore, because of this innate male vulnerability, despite advances in medical care, male loss always exceeds female loss (Wells, 2000).

Natural selection may have favoured the female ability to manipulate M/F since females without such mechanisms would produce fewer grandchildren due to the loss of weaker male infants. Moreover, women who fail to abort male foetuses in times of stress reduce their own odds of survival due to the higher metabolic requirements necessitated by the gestation of a male baby to term. Conversely, a female who aborts a male baby under stressful circumstances fails to invest heavily in what would potentially result in a frail son, and makes herself available to potentially bear a daughter, or a robust son (Wells, 2000).

Indeed, male attrition in pregnancy under stressful conditions may be an inevitable physiological consequence since women who gestate a male embryo require a 10% higher daily energy intake than women who gestate a female embryo, consuming on average 8% more protein, 9.2% more carbohydrates, 10.9% lipids of animal origin and 14.9% lipids of vegetable origin. Male embryos may therefore be more susceptible to energy restriction and therefore more likely to be aborted spontaneously (Tamimi et al., 2003).

This is supported by the fact that M/F is positively correlated with maternal socio-economic status (Shapiro et al., 1968). Dietary calorie intake alone has also been shown to influence M/F, with high intakes increasing M/F (Mathews et al., 2008).

These findings are confirmed by a study that examined various African populations which showed that short maternal stature was also independently related to a lower M/F (Andersson and Bergstrom, 1998). Conversely, big and tall parents have been shown to have a higher M/F (Kanazawa, 2007).

Dwelling ownership has also been shown to affect M/F in a Ugandan sample, with the M/F of mothers who live in owned dwellings at 0.502, as compared with non-owners at 0.458 (Wallner et al., 2012).

However, several large studies have failed to confirm these findings (Erickson, 1976) (Rostron and James, 1977). This discrepancy has been attributed to the relatively short duration of some of the above events since the fetus is very efficient at extracting calories in short-term periods of deprivation (Hytten, 1983). The effect of a population's health on M/F has also been shown on a global scale. It has been shown internationally that M/F is positively correlated with life expectancy and healthy life expectancy. This was demonstrated to hold true for all indicators including individual mortality rate, maternal mortality ratio, under-five mortality rate, adult mortality rate and averaged mortality indices including life expectancy and healthy life expectancy (Dama, 2011).

It has been postulated that socio-economic effects are even more complex, giving rise to an inverted response with a M/F reversal - a so-called *dose-response fallacy*. This hypothesis suggests that M/F increases from low to high as a family's socioeconomic level rises, due to decreasing rates of periconceptual mishaps, resulting in more male survivors. With further rises in socioeconomic conditions, M/F decreases to a more equal gender proportion due to optimal conceptions of both genders (Jongbloet et al., 2001).

Several studies have shown a decreasing M/F in assocation with surrogates of socioeconomic status, such as "descent in the social scale" (Russell, 1936). More recently, in the US population, women married to men listed in various Who's Who volumes have a high M/F

while those listed in social registers have a lower M/F (Mackey and Coney, 1987). This is also quite noticeable at the extreme end of the spectrum, with male billionaires listed in Forbe's having more children with a higher M/F than female billionaires. Furthermore, women married to billionaires had higher M/F than self-made female billionaires (Cameron and Dalerum, 2009).

Interestingly, a study of Caucasian mothers between 1983-2001 that included 48 million births found that married, better educated and younger mothers had a higher M/F, and infant deaths were likelier to be male if the mother was unmarried and young, supporting the Trivers-Willard hypothesis. This is because while younger mothers tend to be healthier, their socio-economic characteristics are often worse than older mothers, and this latter characteristic overwhelms the former (Almond and Edlund, 2007).

Furthermore, it has been shown that working women with higher-earning occupations have a higher M/F and that women working in more traditionally masculine occupations have a higher M/F (Bernstein, 1958). Thus, greater female participation as part of a nation's workforce may potentially influence M/F (Grant and J.T, 2001). Dominant women have also been shown to have higher testosterone levels (Grant and J.T, 2001) and it has been suggested that this may also result in higher M/F ratios in their offspring (James, 2004).

This effect is evident even when social class is taken into consideration (Bernstein, 1958). For example, M/F is increased in royal families (Norton, 1940). Conversely, M/F is lower in men who perform personal services, such as domestic servants, inn-keepers, barmen, waiters, hall porters, barbers and cleaners (McDowall and Britain, 1985).

Furthermore, it has been shown that a stressed parent is likelier to produce a child of the opposite sex (Schuster and Schuster, 1973). This might be due to the fact that anxiety lowers gonadotropin levels in potential mothers (Peyser et al., 1973) and the androgen levels of potential fathers (Kreuz et al., 1972).

Matters are compounded by the opposing hormonal responses to stress of the two sexes. Males lower testicular androgen levels while females increase adrenal androgen levels (Christiansen, 2004). Thus, if both parents are exposed, the hormonal effects on M/F counter each other and may attenuate or even completey nullify any potential effects on M/F (James, 2004).

There are also indications that the Trivers-Willard effect may extend across generations. For example, it has been shown that the reproductive success of individuals born to mothers who had previously had sons is lower than in those who had previously had girls, a Markov variation (Rickard et al., 2007).

Explanations of these findings fall into two broad groups outlined hereunder: the male foetal death mechanism and the reduced male conception theory. The latter is further subdivided into two potential mechanisms: less frequent coitus and poorer sperm quality.

9 Male foetal death

This hypothesis contends that population stressors result in endocrine changes in females that induce the spontaneous abortion of small or weak male fetuses (Forchhammer, 2000; Owen and Matthews, 2003). This accedes to the notion that under such conditions, female offspring are likelier to reach reproductive age and reproduce than male offspring (Trivers and Willard, 1973).

A corollary of the excess male death theory is that males lost due to maternal stress would have been at risk of premature delivery and very low birthweight (<1500g) in the absence of stress. This is confirmed by the finding that the risk of prematurity and very low birthweight increases with maternal stress, and that M/F in such babies is elevated (Hall and Carr-Hill, 1982).

The stress hypothesis is supported by studies that followed up the effects of stressful events on populations. For example, M/F fell in New York City three months after the terrorist attacks of September 11, rather than seven or more months later as would be the case were male conceptions to have been reduced by this event (Catalano et al., 2006).

Similar findings were noted for the same time period, following the above mentioned terrorist attack, in California (Catalano et al., 2005) suggesting that witnessing harm befalling on others induces biological responses that resemble those in the persons harmed (Singer et al., 2004).

Thus, male mortality is higher than female mortality during gestation. Improved socioeconomic conditions and medical care have been shown to be associated with a lowering of prenatal mortality rates. The amelioration of gestational conditions and a reduction in factors that predispose to spontaneous abortion will therefore spare more male than female births, increasing M/F.

This is evidenced in developed countries where the decline in stillbirth rates has been drastic, with, for example, the ratio of stillbirths to live births dropping from 30-40/1000 in the late 1800s in Sweden and Belgium to 10/1000 in the 1970s. This was paralleled by a decline in M/F of stillbirths (Schtickzelle, 1981).

10 Reduced coital rates

One of the defining characteristics of stress in humans is reduced libido, and hence coital rates (Segraves, 1998). Reduced coital rates inevitably reduce the chances of conceptions early in the menstrual cycle. Since it has been shown that conceptions on most fertile days yield

a M/F of approximately 0.492, with a ratio of around 0.536 on other days (James, 1999), reduced coital rates will decrease M/F.

11 Reduced sperm quality

Another effect may be that of the lowering of sperm quality, as already alluded to (Fukuda et al., 1996). This is attributed to the fact that Y-bearing sperm have greater mucosal penetrability, an advantage that is attenuated if motility is reduced, thereby reducing M/F (Pyrzak, 1994).

Life events also affect sperm quality. For example, the recent death of a close family member was associated with a reduction in progressively motile sperm (Fenster et al., 1997).

Natural disasters have similar effects. This was shown one month following the Kobe earthquake in Japan (January 1995), with sperm motility recovering two to nine months after the event (Fukuda et al., 1996).

Factors that influence sperm quality and M/F are complex. Mild stress appears to have overall positive effects on sperm characteristics (Poland et al., 1986). However, high levels of stress negatively influence sperm quality. Stress has been shown to increase prolactin production (Gerhard et al., 1992) and decrease serum testosterone levels (Francis, 1981), and both hormonal changes may interfere with spermatogenesis.

These findings are reinforced by studies demonstrating that soldiers have a lower urinary excretion of testosterone, androsterone, and etiocholanolone prior to combat (Rose et al., 1969). Furthermore, in the early and more stressful part of officer training, plasma testosterone was shown to be suppressed when compared with levels in the later and less stressful part of the course (Kreuz et al., 1972).

Semen volume and normal sperm morphology have been shown to be negatively correlated with levels of psychological stress and low adaptability to stress (Giblin et al., 1988). Work related stress along with stress related to family dynamics or those at the workplace have also been associated with lower sperm morphology and vitality (Gerhard et al., 1992).

Furthermore, it has already been pointed out that stress may reduce the frequency of sexual activity. Since it has also been shown that sexual abstinence may result in a higher proportion of X-bearing sperm (Hilsenrath et al., 1997), all of these factors further predispose to female conceptions.

Overall, sperm motility appears to be the factor most affected by stress (Bigelow et al., 1998). For example, in infertile couples undergoing *in vitro* fertilisation, sperm motility declined at the time of oocyte retrieval, ostensibly due to the perception of the importance of producing an adequate semen sample for the fertilisation process (Clarke et al., 1999).

All of this is compounded by studies that show that sperm motility is secularly progressively decreasing. For example, sperm volume, concentration, count and total sperm motility was unchanged for the period 1988 to 1996, while rapid progressive sperm motility decreased by a mean of 0.95% per annum (Zorn et al., 1999).

12 Other man-made stress

The reunification of Germany in 1990 resulted in the temporary economic collapse of the East German region in 1991, and this was associated with a significant decline in M/F (Catalano, 2003).

The unemployment rate was specifically used as a surrogate for ambient economic stressors less extreme than collapsing national economies, and was also shown to induce an excess of male fetal deaths (Catalano et al., 2005).

13 Miscellaneous parental influences

M/F is decreased in mothers with anorexia nervosa and bulimia nervosa, while binge eating disorders increased M/F (Bulik et al., 2008).

It was indirectly shown as far back as 1932 that male preference was associated with increased M/F (Winston, 1932). This was confirmed by the fact that in response to the question "what sex for the neonate do you prefer" a significant correlation was found between sex preference and the gender of newborn (Emamghorashi et al., 2011).

Handedness also affects M/F. M/F is lower in the offspring of left-handed parents than in that of right-handed people (James, 1986a). Parental attractiveness has also been shown to be negatively correlated with M/F (Kanazawa, 2007).

Two-parent care has also been shown to increase M/F, a finding that was first noted in 1874 (Darwin, 1874). Moreover, in polygynous arrangements, when co-wives co-habit in a harem, M/F is increased whereas when co-wives have separate dwellings and are visiting by their husband, M/F is reduced (James, 1995).

Time to achieve pregnancy also influences pregnancy. A longer interval to achieve a pregnancy increases M/F, and this is in accordance with the hypothesis that poorly penetrable cervical mucus reduces fertility and increases the chance of a smaller and faster Y-bearing sperm achieving conception (Smith et al., 2005).

14 Seasonal variation

Cold weather is an environmental stressor, acting directly on those who do not have adequate access to shelter. Such conditions also influence indirectly, stressing

populations by disrupting economies (Catalano et al., 2008).

It has also been shown that cold weather exacerbates the effect of other stressors, particularly in unseasonally cold summer months, since such conditions mitigate against restorative behaviours (Catalano et al., 2008). According to the Trivers-Willard hypothesis, both direct and indirect mechanisms may cause pregnant females to abort frail male fetuses, lowering M/F. Indeed, European data for the period 1865-2003 shows an increase in M/F in warm years (Helle et al., 2009).

More cogently, a study of the Sami people (the only indigenous Scandinavian race) showed that a 1°C increase in mean temperature yielded 1% more sons annually (Helle et al., 2008). Interestingly, in this same population, annual mean temperature and birth rate seem unrelated (Helle and Helama, 2007).

Temperature influences on M/F may have long-term effects. It has been shown not only that M/F is influenced by ambient temperature, but also that males from cold-stressed cohorts who have experienced cold weather *in-utero* could have, on average, longer life expectancies. This has been calculated as an average decrease in male life-span by 14 days per 1°C increase from one year to the next among those who survived to one year of age (Catalano et al., 2008).

15 Environmental toxins and occupations

A plethora of environmental toxins have been implicated as affecting M/F with parental exposure, but only a representative sample will be outlined hereunder.

Decreasing M/F The industrial accident that occurred in Seveso (Italy) in 1976 exposed a large population to dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin), widely considered the most toxic man-made substance. Among many other local effects (Bertazzi et al., 1998), M/F declined significantly with paternal exposure, even in births in 1996 (Mocarelli et al., 1996), in a dose-effect relationship (Mocarelli et al., 2000).

Polychlorinated biphenyl (PCB) compounds (banned in 2001) were widely used as coolant fluids and dielectrics in industry prior to this time. The parental consumption of PCB contaminated rice oil (del Rio Gomez et al., 2002) and the maternal consumption of contaminated fish (Weisskopf et al., 2003) has been shown to decrease M/F.

Pesticide exposure has similar effects, including the paternal exposure to the nematocide 1,2-Dibromo-3-chloropropane (Goldsmith, 1997), pesticide applicators (Garry et al., 2003) and hexachlorobenzene (Jarrel, 2002). It has been speculated that these effects may be hormonally induced (James, 1987b). For example, it has been shown that exposure to the above nematocide increases paternal gonadotrophin levels (Whorton et al., 1979), which would in turn lower M/F.

Methylmercury contamination of Minemata bay in Japan in 1955 to 1959 and the resultant maternal exposure to contaminated fish was also shown to reduce M/F (Sakamoto et al., 2001)

Parental exposure to air pollution from incinerators (Williams et al., 1992) and cigarette smoking (Fukuda et al., 2002) also decreases M/F. Smoking reduces maternal oestrogen levels (MacMahon et al., 1982) and this has been attributed to lower M/F (James, 1987b). However, a more recent study showed that maternal smoking increased M/F, more so in primiparous mothers (Beratis et al., 2008).

The paternal exposure to chemicals in the carbon typesetting occupation (Milham, 1993); the maternal exposure to clomiphene citrate in infertility treatment (Jarrel, 2002); the paternal exposure to high voltages and the maternal exposure to non-ionising radiation (James, 1997) have all been implicated as decreasing M/F. Males in these occupations have been shown to have low testosterone levels (Grajewksi et al., 2000), and it has been speculated that the latter may cause the former (James, 2004).

Male exposure to lead, alcohol (Dickinson and Parker, 1994), boron (Chang et al., 2006), vinclozolin (James, 1997), dibromochloropropane (Potasjnik and Yanai-Inbar, 1987), non-Hodgkin's lymphoma (Olsson and Brandt, 1982), children of men who later go on to contract prostatic cancer (Hill et al., 1985) and the development of insulin-dependent diabetes mellitus (Rjasanowski et al., 1998) have all been shown to decrease M/F.

These factors would all seem to suggest that industrially (or otherwise) contaminated environments reduce M/F. Indeed, in Japan, the male/ female ratio of fetal deaths (after 12 weeks) was reported to be increasing from the 1970s: this trend suggests a particular prenatal vulnerability of the male fetus in the face of adverse prenatal conditions (Mizuno, 2000).

However, in Finland a study over the period 1751 to 1997 has demonstrated that the decline in M/F preceded the onset of industrialization and the introduction of pesticides and hormonal drugs (Vartiainen et al., 1999).

HLA markers may also affect M/F. It has been shown that men with rheumatoid arthritis and HLA B15 had the lowest M/F (Astolfi et al., 2001) and the lowest testosterone levels (Ollier et al., 1988), and it has been speculated that the latter may mediate the former (James, 2004).

Certain occupations may also affect M/F. Male

drivers have been shown to have poor sperm quality and low testosterone levels and this has been linked with a low M/F (Dickinson and Parker, 1994). Divers also have low M/F (Rockert, 1977, Lyster, 1982) and they have also been shown to have lower testosterone levels (Rockert and Haglid, 1983). Similarly, pilots of high-performance aircraft and spacecraft also exhibit low M/F (Snyder, 1961) and low testosterone to gonadotrophin ratios (Strollo et al., 1998). It has been suggested that low M/F in all of these associations is related to low testosterone levels (James, 2004).

Increasing M/F An increase in M/F was noted in localites close to a steel foundry (Lloyd et al., 1984), close to natural gas (Saadat et al., 2002) and to the petrochemical industry (Yang et al., 2000).

The overall effect of a toxin is probably modulated by a number of factors, which include parental age at the time of exposure, the total level of exposure and whether the exposure was maternal or paternal (Mackenzie et al.,).

Exposure to ionising radiation has been shown to elevate M/F. Radiation increases the incidence of lethal malformations, affecting female pregnancies more than male pregnancies. Hence, when both parents are equally exposed, fertility decreases while skewing birth rate in favour of males (Scherb and Voigt, 2011).

16 Placental pathology

M/F is increased in pregnancies with abruptio placenta, placenta praevia, fatty liver and toxaemia of pregnancy. On the other hand, M/F is decreased in pregnancies with placenta accreta and ectopic pregnancy.

A review of such placental pathology postulated that M/F deviations are caused by abnormal hormone concentrations periconceptually, which persist and are partially responsible for the abovementioned pathologies (James, 1995).

17 General temporal trends

Non-random, slight but highly statistically significant secular trends in M/F were first described in 1955 (Gini, 1955). Due to the non-trivial numbers usually invoked, relatively small changes, when appropriately tested, yield high significance levels. A large sample of the available reported national datasets are listed in table 1.

Table 1: Historical studies of secular trends in M/F.

(Chahnazarian, 1990)	Japan	1950-70	Increasing
(Schtickzelle, 1981)*	Belgium, Sweden, England and Wales	1900-90	Increasing
(Chahnazarian, 1990)	Taiwan	1945-90	Increasing
(Ulizzi, 1983; De Bartolo, 1985)	Italy	1930s-90	Increasing
(Chahnazarian, 1990)	England, Wales, US Caucasians, Japan	1970-90	Decreasing
(Aubenque, 1989)†	France	1800-90	Decreasing
(Chahnazarian, 1990)	Australia	1900s	No trend
(Chahnazarian, 1990)‡	Sweden	1750-00	Increasing
(Moeller, 1993)	Denmark	1951-95	Decreasing
(van der Pal-de Bruin et al., 1997)	Netherlands	1950-94	Decreasing
(Allan et al., 1997)	Canada	1930-90	Decreasing
(Allan et al., 1997)	USA	1970-90	Decreasing
(Feitosa and Krieger, 1992)	Uruguay, Chile, Argentina, Brazil, Bolivia, Peru, Paraguay, Ecuador, Venezuela, Colombia, and Costa Rica	1967-86	Decreasing
(Imaizumi and Murata, 1981)	Japan	1900-78	Increasing
(Ulizzi and Zonta, 1995)	Italy	1930-90	Increasing
(Vartiainen et al., 1999)	Finland	1751-20	Increasing
(Vartiainen et al., 1999)	Finland	1920-97	Decreasing

*This study also failed to find trends for Australia and the United States.
†This study showed that the variation noted was more closely associated with a variation in the male birth rate than in the female birth rate.
‡No trends for the period 1900-1990.

Overall, the most striking findings have been a decline in M/F over the second half of the 20th century in various industrialised countries (Davis et al., 1998).

A few papers have reported aggregate country

datasets. The largest prior to this author's publications summarised secular trends in M/F five populations: United States Caucasians and Blacks, Australia, Japan and Taiwan during this 20th century and for a few countries (Sweden, France, England and Wales), for even longer periods (Chahnazarian, 1988).

However, a study from Malta was the first to identify secular trends using continent-wide data from a World Health Organization (WHO) dataset. Grech et al. studied secular trends in gender ratios for live births over the second half of the 20th century. These included 12,7034,732 North American and 15,7947,117 European live births.

This study showed a highly significant overall decline in male births in both Europe and North America ($p<0.0001$), particularly in Mexico ($p<0.0001$). Interestingly, in Europe, male births declined in North European countries (latitude>40 degrees, $p<0.0001$) while rising in Mediterranean countries (latitude congruent with 35-40 degrees, $p<0.0001$). These trends produced an overall European male live birth deficit 238,693 and a North American deficit of 954,714 for the period under study (total male live birth deficit 1,193,407). This study concluded that were no reasonable explanation/s for the observed, and the causes for these trends may well be multifactorial (Grech et al., 2003).

18 Latitude gradients in M/F

The same Maltese group were also the first to note a latitude gradient in M/F. Annual data was obtained for European countries from official WHO publications and manually input into a spreadsheet for the period 1990-1995.

European countries were banded by latitude. Southern countries (latitude $35-40°$) included Bulgaria, Greece, Italy, Malta, Portugal, and Spain. Central Europe ($40-55°$) included Austria, Belgium, Czech Republic, France, Germany, Hungary, Ireland, Luxembourg, Netherlands, Poland, Romania, Switzerland, and the United Kingdom. Nordic countries ($> 55°$) include Denmark, Finland, Iceland, Norway, and Sweden.

Analysis of European births showed a much higher ratio of male births in the south of Europe than in the north ($p<0.0001$). At this stage, the authors speculated that M/F might be somehow influenced by a factor related to latitude, such as temperature (Grech et al., 2000).

The same authors acquired annual data on male and female live births from WHO for the North American continent for 1958-97 and for European countries for 1950-99 (courtesy of Mie Inoue from WHO). Overall less than 3% of data were missing.

European countries were banded as above (Grech et al., 2000). North America was divided by latitude into Canada ($> 50°$), the United States ($30-50°$), and Mexico ($< 30°$).

Significantly, more boys were born in southern countries than in central Europe ($p<0.0001$) or the Nordic countries ($p=0.003$) countries (1950-99), confirming the first study (Grech et al., 2000). Trend analysis was highly significant ($p<0.0001$). All had a M/F <0.515, with a resultant male birth deficit of 12744 in the Mediterranean, 212,780 in central Europe, and 13169 in the Nordic countries (a total deficit of male births 238,693).

However, the converse latitude gradient was evident in the North American continent. A low M/F was found in Mexico, a higher ratio in the United States, and an even higher ratio in Canada (trend analysis $p<0.0001$). All had a M/F<0.515, with a resultant male birth deficit of 21,993 in Canada, 410,932 in the United States, and 521,789 in Mexico (total deficit 954,714). In the two continents, the total male birth deficit was 1,193,407 live births (for the years 1958-97).

In summary, in Europe, significantly more male babies were born in southern latitudes than in northern latitudes, whereas the reverse was found in North America. The authors were unable to explain these findings, which do not support a latitude related effect (Grech et al., 2002).

19 Conclusion

A veritable plethora of factors influence M/F in order to finally reach a value that approximates 0.515. It is therefore very true to say that "the persistent, exactly equal difference in this proportion at birth, and even its relatively small variations have provided food for thought for theologians, mathematicians, social scientists and biologists ever since the first calculations were made, even up to the present day" (Brain and Jaisson, 2007). Indeed, the factors that influence M/F are dynamic areas of research with over a thousand published papers to date. Doubtless, many more factors that influence M/F will be found through the utilisation of ever larger and pooled datasets with longer time frames.

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News Article

COST Action Chemistry Conference on Supramolecular Chemistry in Water

David C. Magri[1]
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1 Introduction

Scientists and chemists from 18 countries gathered in Malta for the 3rd Scientific Meeting on Supramolecular Chemistry in Water between the $9-11^{th}$ of November 2013 at the Old University Building on St Paul Street in Valletta. The conference commenced Saturday morning with a presentation by Prof. Kay Severin of the École Polytechnique Fédérale de Lausanne, Switzerland on the principles of constructing chemosensors for detecting small chemical species such as lithium ions and caffeine. The Sunday morning plenary lecture was given by Prof. David K. Smith of the University of York, UK on nano-sized self-assembled arrays for binding DNA and heparin, a common blood thinner. The final plenary lecture on Monday morning was presented by Felix Müller of Evonik Industries on the practical applications and commercial successes of using detergents for removing bad odours in laundry and in the air. During the conference over 25 oral communications and 28 posters were presented including five research projects from University of Malta students. The local organizing commitee consisted of the host academic Dr. David Magri, a senior lecturer in the Department of Chemistry, and the staff of the Conference Support Unit at the University of Malta under the coordination of Lucienne Bugeja. The scientific program was organised by a consortium of researchers within COST Action CM1005. There were over 90 participants in attendance.

Figure 1: Researchers during a coffee break in the hallway of the Old University Building on St. Paul Street in Valletta at the 3rd Scientific Meeting on Supramolecular Chemistry in Water between the $9-11^{th}$ of November 2013.

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News Article

1st Lecturer's Mini-Symposium

David C. Magri[1]
[1]Department of Chemistry, Faculty of Science, University of Malta

1 Introduction

Academic staff from 18 different departments and institutes gathered for a forum to present and discuss their research interests. The research disciplines spanned science, art, entrepreneurship, social studies and engineering. The symposium was held on January 30th, 2014 in the Conference Room at University House, Msida campus, University of Malta from 3:00 to 5:30 pm. The meeting consisted of two sessions of 11 speakers each presenting a brief 4 minute overview of their research and professional interests with an intermission for socialising and networking between colleagues. A kaleidoscope of topics were presented including the design of intelligent transport systems, lanthanide crystal engineering, youth and social well-being studies, surface coatings for mechanical gears and historic artifacts, dance studies, genetically modified fruit flies for studying neuromuscular disease, creativity and entrepreneurship, fluorescent intelligent molecules, input-output economic analysis, antioxidants, auxetic materials, gender studies, blood disorders and food safety. The symposium promoted multi-disciplinary collaborations while developing new friendships. Participants were invited based on contributions to research, society and gender balance. The symposium was financially supported by the Malta Chamber of Scientists.

List of Participants and Order of Presentations

L1: **David Magri** - Department of Chemistry
L2: **Giuseppe Di Giovanni** - Editor-in-Chief of Xjenza/Department of Physiology & Biochemistry
L3: **Joanne Cassar** - Department of Youth & Community Studies

Correspondence to: D. Magri (david.magri@um.edu.mt)

Figure 1: The poster is compiled from figures from the various presentations given at the Lecturer's Mini-Symposium on January 30th, 2014 in the Conference Room at University House, Msida campus, University of Malta.

L4: **Ruben Cauchi** - Department of Physiology & Biochemistry
L5: **Daniel Vella** - Department of Metallurgy & Materials Engineering
L6: **Joanne Butterworth** - Department of Dance Studies, School of Performing Arts
L7: **Vasilis Valdramidis** - Department of Food Studies & Environmental Health
L8: **Maria Attard** - Department of Geography/Institute of Climate Change & Sustainable
L9: **Joseph Borg** - Department of Applied Biomedical Science
L10: **Bridget Ellul** - Department of Pathology
L11: **Edward Duca** - Editor of Think
Networking Break
L12: **Alex Felice** - President of the Malta Chamber of Scientists/Department of Physiology & Biochemistry
L13: **JosAnn Cutajar** - Department of Gender Studies/Cottonera Research Centre
L14: **Ulrich Baisch** - Department of Chemistry

L15: **Leonie Baldacchino** - Edward de Bono Institute for the Design and Development of Thinking
L16: **Neville Vassallo** - Department of Physiology & Biochemistry
L17: **Ann Zammit** - Department of Metallurgy & Materials Engineering
L18: **Ian Cassar** - Department of Economics
L19: **Daphne Attard** - Metamaterials Unit
L20: **Kenneth Scerri** - Department of Systems & Control Engineering
L21: **Claire Shoemake** - Department of Pharmacy
L22: **Richard Muscat** - Pro-Rector of Research & Innovation/ Department of Physiology & Biochemistry

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News Article

Science in the House 2013

David C. Magri[1]
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1 Introduction

On Thursday September 26 at 4 pm the inauguration of Science in the House opened at the Grand Master's Palace, Valletta. A poster exhibition entitled "Science in the House" was inaugurated under the auspices of the Office of the Speaker at the House of Representatives in the Grand Master's Palace, Valletta. The exhibit was organised by Dr. David Magri (University of Malta) on behalf of the consortium "Researchers' Night - Science in the City"

The researchers were greeted by the Deputy Speaker the Hon. Censu Galea in the presence of Project Coordinator for Science in the City and Chair of the Malta Chamber of Scientists Prof. Alex Felice, University of Malta Pro-Rector for Research & Innovation Prof. Richard Muscat, Chief Executive of the Research Trust (RIDT) Wilfred Kenely, and Members of Parliament.

Figure 1: Scientists and researchers from the University of Malta, parliamentarians and journalists at the inauguration of the 2013 Science in the House at the Grand's Masters Palace in Valletta.

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Figure 2: (left to right) Chair of the Malta Chamber of Scientists Prof. Alex Felice, Chief Executive of the Research Trust (RIDT) Wilfred Kenely, Deputy Speaker The Hon Censu Galea and University of Malta's Pro-Rector of Research and Innovation Prof. Richard Muscat discussing and viewing the poster exhibition.

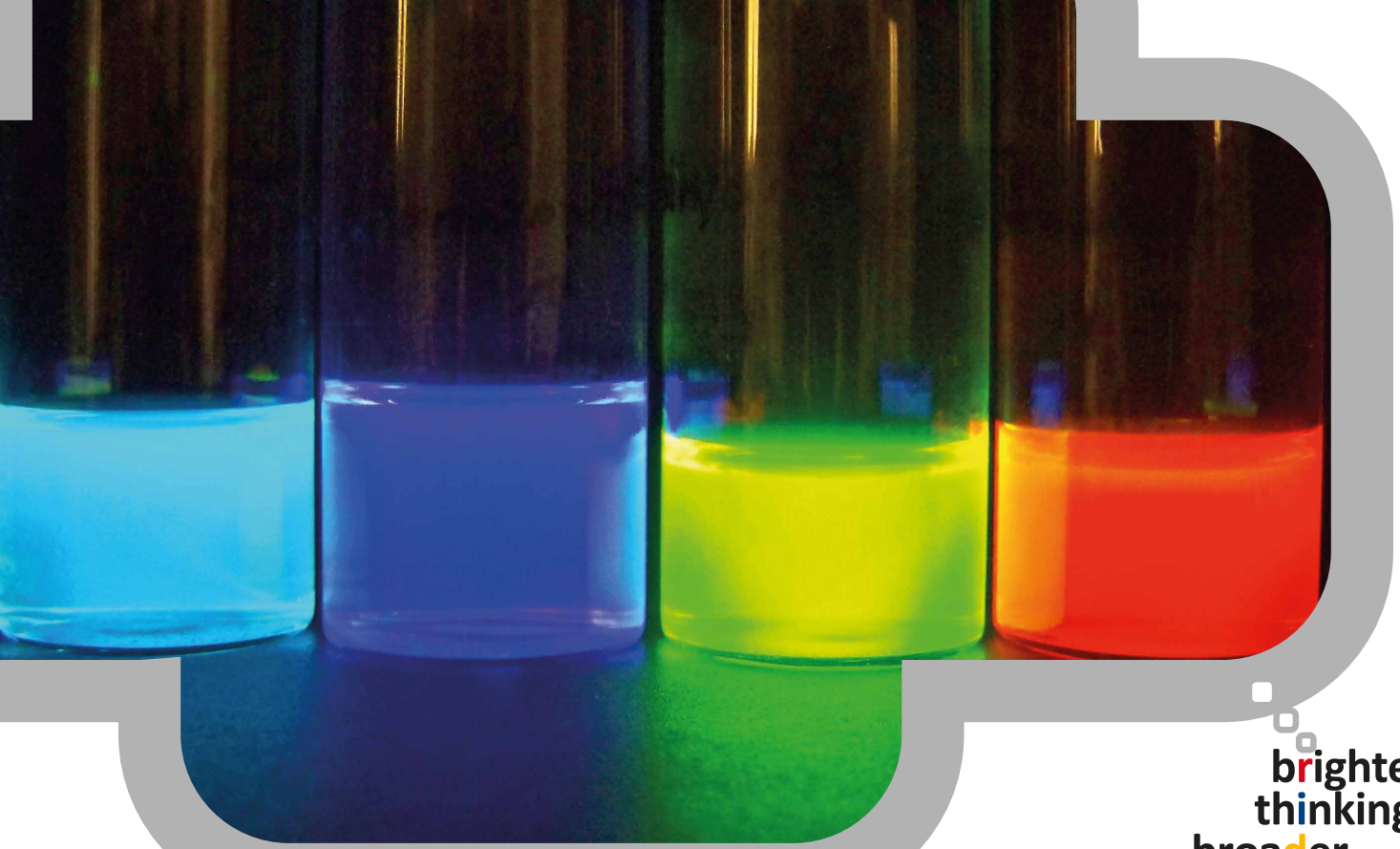
Following the opening speeches, the Deputy Speaker was taken for a tour of the posters. A set of 12 posters were consistent with the theme "Today's Science, Tomorrow's Jobs" and the slogan of the Research Trust "Brighter thinking, broader future". Each poster showcase a particular research study, the team involved in the research, and the societal contribution the research may make our lives. The exhibition aims to provide a unique opportunity for Members of Parliament and the public to meet scientists and learn how the research is contributing to a better quality of life.

The "Science in the House" exhibition will be open for public viewing tomorrow (today) during Science in the City from 6.00pm to 9.00pm and on the 5th October during Notte Bianca. During the week of the 30th September, the exhibition will be open for viewing by the parliamentarians.

Science in the House is a forum for networking and cause for celebration involving Maltese research scientists, Representatives of the House and Members of Parliament. It is also a poster exhibition highlighting

Figure 3: Sacha Dunlop (left) and Katrina Grech (second from left), research students under the supervision of Sandro Lanfranco, presenting their poster to the Deputy Speaker the Hon Censu Galea (second from right) and the Project Coordinator for Science in the City and Chair of the Malta Chamber of Scientists, Prof. Alex Felice (right).

some of the exciting scientific research currently conducted in Malta, particularly at the University of Malta. The event encourages and supports the achievements of Malta's early-stage research scientists, engineers and technologists - arguably the "creative engines" of future innovation and development in Malta. During the following week the exhibition is open for viewing by the parliamentarians and afterwards left on display in the Grand Master's Palace over the Notte Bianca festival, thus allowing the general public including students, parents and tourists to be more aware of local research endeavors. The event was sponsored by the Malta Chamber of Scientists, the Research, Innovation & Development Trust (RIDT) and the University of Malta.



Dr. David C. Magri

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Intelligent Molecules

for Medical Diagnostic Applications

The Research

As "molecular engineers", chemists perform chemical transformations to build rationally designed molecules with useful functions. Research is currently being carried out to develop intelligent molecules that operate as logic gates for medical diagnostics applications. For example, molecules can be designed to report the presence of one or more chemical species in a vial by emitting bright fluorescent colours after irradiation with light from a lamp.

How it Makes Our Lives Better

With the continual rising cost of health care, the creation of intelligent molecules able to simultaneously detect many analytes at once for a specific disease could help doctors to diagnose patients more effectively, and reduce the waiting time for laboratory test results.

The Research Team

Research on intelligent luminescent sensors and molecular logic gates is being carried out by Dr. David C. Magri and postgraduate and undergraduate students of the Department of Chemistry in the Faculty of Science at the University of Malta.

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Osteoporotic bone is more porous and weaker, making it prone to fractures with deterioration of vertebral support and loss of weight.

Bone Health
and Osteoporosis

Prof. Angela Xuereb • Ms. Melissa Formosa

brighter thinking broader future

The Research

Osteoporosis is a bone disease that negatively affects the bone structure. As we grow older, deterioration of the bone structure affects our vertebrate such that we get shorter. Our bone mass starts deteriorating after we reach the age of 30. Although this affects both men and women, the rate of bone deterioration is faster for women after menopause. Although not all fractures are the result of loss of bone mass, the lifetime risk of a fracture resulting from such a condition is 40% for women and 13% for men. The most common fracture sites are the spine, hip, wrist and humerus. The bone mineral density (BMD) measurement test is used to diagnose osteoporosis and to predict fracture risk. Lifestyle factors (such as smoking and low Calcium intake), coexisting medical diseases or prolonged use of glucocorticoids are major contributing factors in the development of osteoporosis and fractures, which are both highly hereditary.

How it Makes Our Lives Better

The results of this study will possibly aid in the early diagnosis of osteoporosis and fracture, as well as in the development of personalised medicine. Early detection is the key in preventing unnecessary suffering and escalation of health care costs.

The Research Team

The research is being conducted by Professor Angela Xuereb and research staff and students of the Department of Applied Biomedical Science in the Faculty of Health Sciences at the University of Malta.

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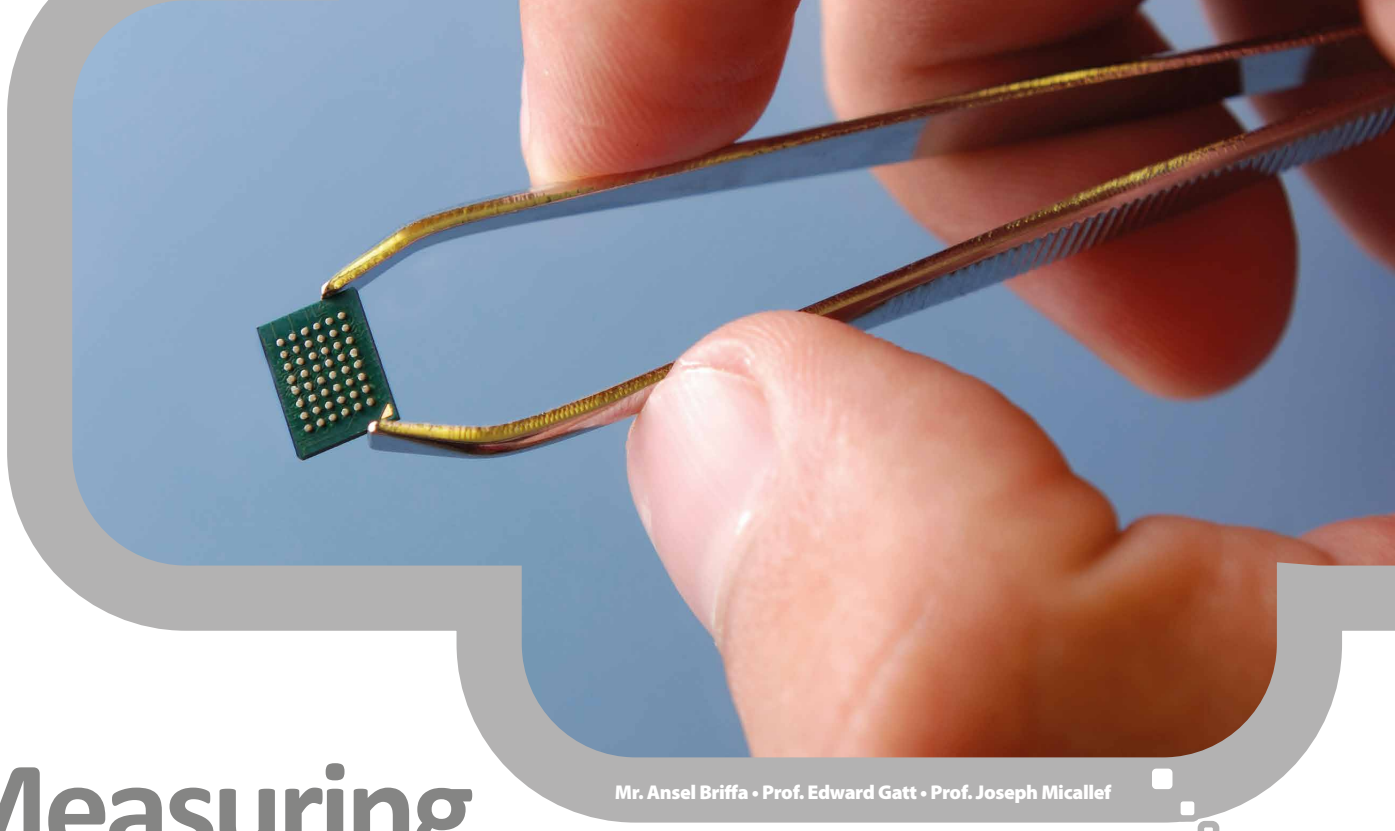

Mr. Ansel Briffa • Prof. Edward Gatt • Prof. Joseph Micallef

Measuring Acceleration

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The Research

Micro-electro-mechanical systems (MEMS) are very small devices on the micro scale (that is one millionth of a metre), although they range in size from a few micrometres to a few millimetres. These consist of two units: the central unit, which is used to process data and the mechanical sensor, which interacts with the surroundings. The current research focuses on a type of MEMS known as an accelerometer. A MEMS accelerometer is a device that measures proper acceleration (or rate of change of velocity). There are two types of accelerometers: static due to gravity, and dynamic, which operates based on movement or vibration. Accelerometers are now a common component in most smart-phones and tablets.

How it Makes Our Lives Better

Demands for low-cost accelerometers have increased rapidly in recent years which augment our day to day life. Accelerometers are also used in navigation, transport, building and structural monitoring, medical applications and consumer electronics. The scope of this research was to design and fabricate novel three-axis accelerometers having separate masses using an industrial fabrication process.

The Research Team

This research was conducted by Mr. Ansel Briffa under the supervision of Professor Ing. Edward Gatt and Professor Ing. Joseph Micallef of the Department of Microelectronics & Nanoelectronics in the Faculty of Information and Communication Technology at the University of Malta.

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Newly Discovered Brain Area Offers Hope to Stopping Nicotine Addictions

Prof. Giuseppe Di Giovanni

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The Research

Smoking is the leading preventable cause of disease, disability and death in the world with approximately 5 million deaths each year. So why do people smoke? It is a well known fact that nicotine is one of the most addictive substances known to mankind because it stimulates feelings of pleasure. Similarly to other addiction drugs, such as cocaine, heroin, and marijuana, nicotine increases the levels of the neurotransmitter dopamine, which affects the brain pathways that control reward and pleasure. It is for this reason that it is difficult for people to stop smoking as the brain wants to avoid feeling bad after such a pleasurable experience.

How it Makes Our Lives Better

Are there effective treatments for tobacco addiction to help people stop smoking? One consideration is nicotine-replacement treatments such as bupropion and varenicline. An alternative is a vaccine called NicVax. Our research suggests that the brain area called the lateral habenula might be a new drug target for the treatment for nicotine cessation. We hope to find a new effective drug soon.

The Research Team

This research is being carried out under the supervision of Professor Giuseppe Di Giovanni at the Department of Physiology and Biochemistry in the Faculty of Medicine & Surgery at the University of Malta.

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Dr. Sandro Lanfranco • Mr. Edwin Lanfranco

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Plant Diversity and Natural Heritage

The Research

Endemic plants – There are approximately twenty species of plants which are unique to the Maltese islands and not found anywhere else on Earth. Such plants are therefore of considerable value on both a local and global scale. There are no detailed studies on the life-cycles and reproductive capacity of these so called endemic plants and these are vital for their effective conservation. One such plant is the Maltese Everlasting (Sempreviva ta' Ghawdex; Helichrysum melitense), a plant that is only found along the western coast of Gozo as species that has a very low fertility.

Shoreline algae – The gently sloping rocky shores of the Maltese Islands harbor a wide variety of algae. Why are these algae so important?

- They help reconstruct past ecosystems and monitoring sea-level changes
- They respond to changes in water chemistry to rapidly help assess the pollution load in seawater without resorting to chemical tests.

How it Makes Our Lives Better

Plants are indicators of the 'state of health' of our natural ecosystem. Unfortunately much of the natural environment in the Maltese Islands has been degraded by human activity, so monitoring the 'state of health' of natural ecosystems is of prime importance for one to be able to assess

- the value of habitats,
- the impact of damage caused by human activity and
- the effectiveness of conservation measures

The Research Team

This research is being conducted by the Plants and Algae Research Group composed of Dr Sandro Lanfranco, Mr Edwin Lanfranco and a number of postgraduate and undergraduate research students at the Department of Biology in the Faculty of Science at the University of Malta.

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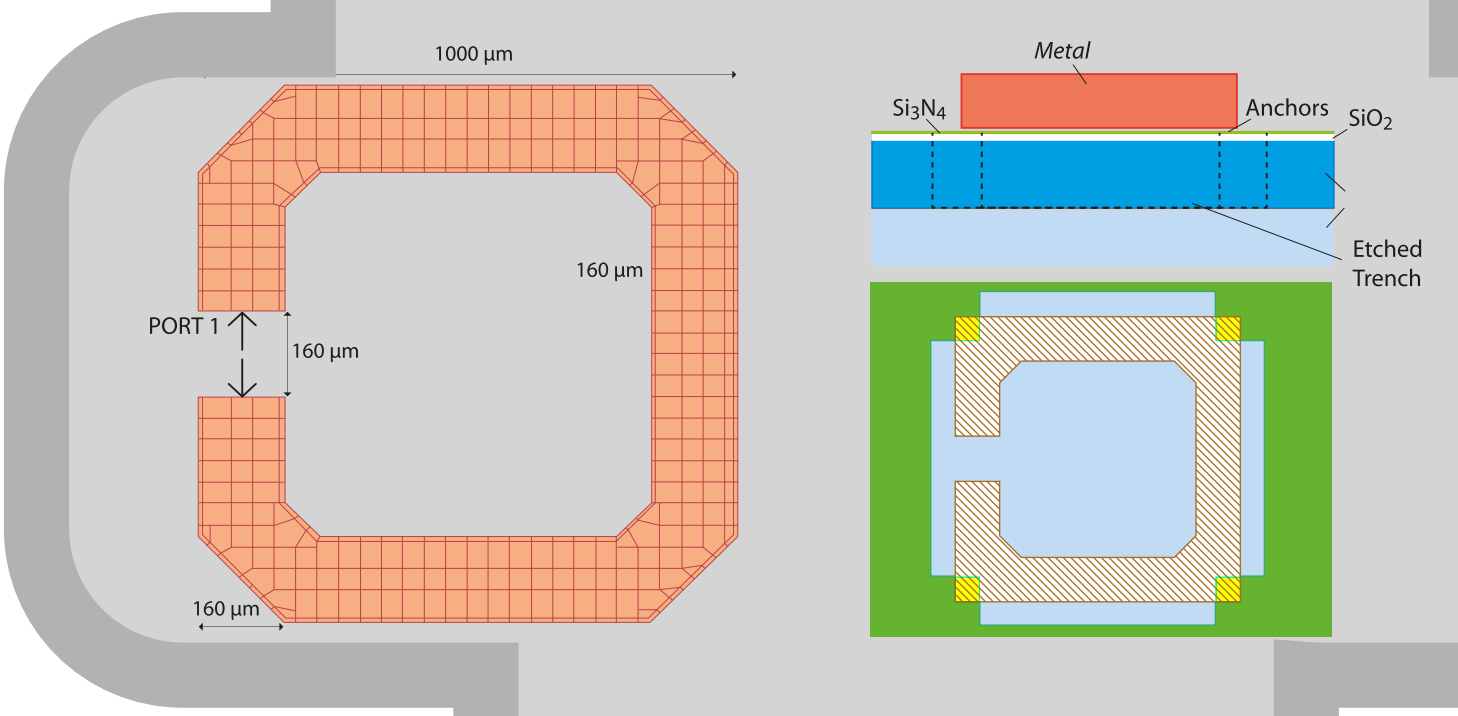

Dr. Owen Casha • Mr. Daniel Zammit

Increasing Mobile Phone Efficiency

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broader future

The Research

Wireless communication devices, such as mobile phones, need a particular type of circuit that generates a high number of electrical signals per second in order to send and receive information. The problem with traditional electronic circuits is that they are inefficient resulting in unnecessary battery-power consumption. In order to make this circuit more efficient, Micro-Electromechanical Systems or MEMS (these are small devices on the scale of a human hair) are employed as a key component. Our research has resulted in a significant improvement over traditional circuit implementations.

How it Makes Our Lives Better

Power consumption is a very important parameter in any mobile device. With MEMS technology, our batteries would last longer and apart from extending the operational time of the device, the amount of charging cycles required would also be reduced.

The Research Team

This research was carried out by Daniel Zammit at the Department of Microelectronics & Nanoelectronics in the Faculty of Information and Communications Technology, under the supervision of Dr. Owen Casha, through a scholarship scheme offered by STMicroelectronics, Malta.

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RIDT

Dr. Godfrey Grech • Prof. Christian Scerri • Dr. James DeGaetano

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Better Quality of Life for Cancer Patients

The Research

Understanding the cause of disease requires systematic methodology, interrogating cellular communication. Interference in normal communication within cells and between cells is the source of irregular cellular growth and multiplication, hence the initiation of a new cancerous growth. Recent research has identified novel mechanisms of cancer initiation with potential new therapeutic targets. Directing specific therapies and deriving knowledge on response to the therapy in cancer patient groups is the key for cure.

How it Makes Our Lives Better

Our research uses research-derived knowledge to design methods to classify patients into therapeutic groups and predict therapy outcome within such groups. These platforms enhance the quality of life of cancer patients, promoting personalised medicine.

The Research Team

Cellular Biology and Genetics Research using cellular models and patient material is headed by Dr. Godfrey Grech, Department of Pathology at the University of Malta. Clinical Information and patient material are coordinated by Professor Christian Scerri (Consultant, Genetics Clinic, Mater Dei Hospital) and Dr. James DeGaetano (Consultant Pathologist, Mater Dei Hospital).

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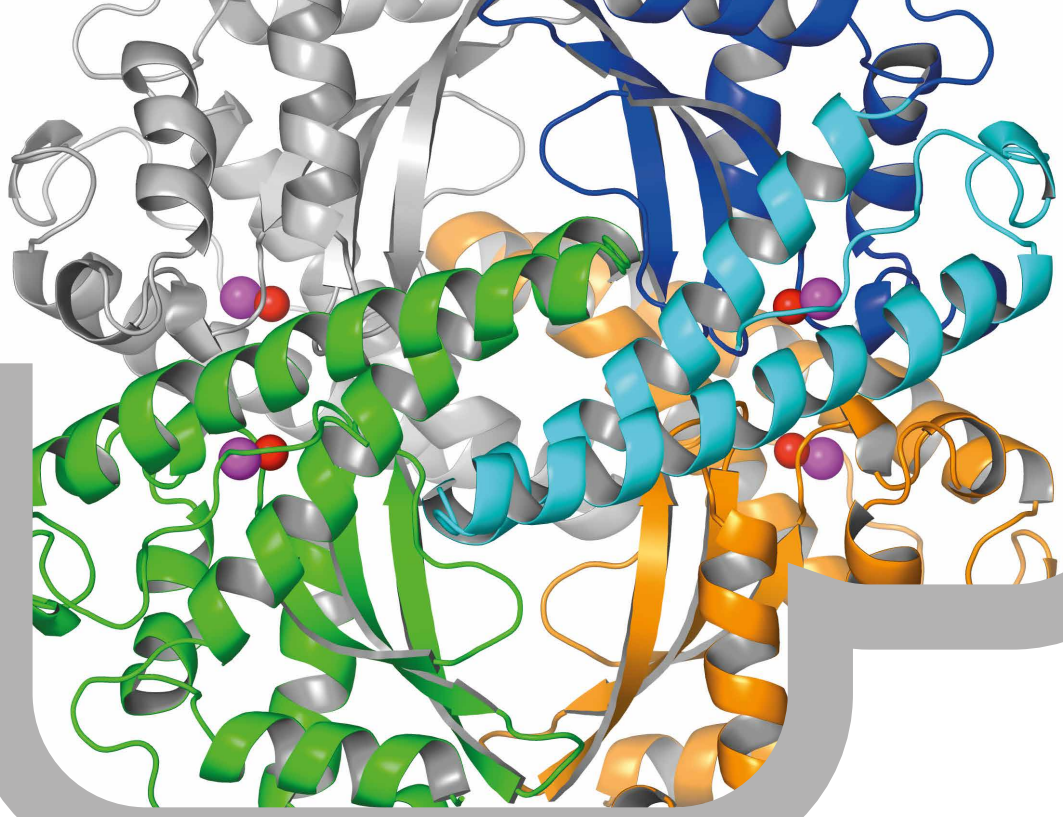

Manganese Superoxide Dismutase structure determined in our laboratory

Prof. Gary J. Hunter • Dr. Thérèse Hunter

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The Structure of Protein Molecules

The Research

Proteins are the workforce of the body. There are many different types of proteins in our body: they digest our food, give us energy, make up our muscles, protect us from infection. Our cells synthesize proteins all the time and when something goes wrong with the way they function it may have fatal consequences. A protein is made up of thousands of atoms and is in itself as complex as the solar system. In the Department of Physiology and Biochemistry, a team of scientists is working on the purification, biochemical characterisation, and determination of the molecular structure of proteins.

How it Makes our Lives Better

Determining the structure and mechanism of function of a protein enables researchers to understand the basis of disease and to design drugs that will improve our quality of life.

The Research Team

A team of enthusiastic scientists supervised by Professor Gary J. Hunter and Dr. Thérèse Hunter is working together in the Laboratory of Biochemistry and Protein Science at the University of Malta. Collaborations have been established with colleagues at leading laboratories including the Astbury Centre for Structural Molecular Biology, UK, the CEA, France and the University of Tokyo, Japan.

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Prof. Alex Felice • Dr. Joseph J. Borg • Mr. Clint Mizzi

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Hereditary Blood Disorders and Gene Control

The Research

The Laboratory of Molecular Genetics conducts basic research on important themes in molecular biology and genetics. These include the blood profiling for rare and other genetic disorders in the Maltese population and the molecular characterization of mechanisms and pathways leading to the developmental switching of haemoglobin in humans. Important discoveries from our lab have led us to speculate on a small set of genes and proteins that seem to be essential in regulating the development and maturation of red blood cells. This is all coded in our DNA, and variations in the DNA can lead to disorders, which we consistently scan and look out for with our laboratory equipment and instrumentation. A field better known as Bioinformatics, handles large datasets of generated numbers and results to make sense and analyze scientifically the effects.

How it Makes Our Lives Better

The impact of this research is felt by those who essentially are inflicted with a genetic disorder, such as beta thalassaemia and sickle cell disease since it can potentially cure their illness. Moreover, this research serves as a model for other analogous research being carried out elsewhere as it principally demonstrates how different life science themes can merge and cooperate together for a successful outcome.

The Research Team

The research team is headed by Professor Alexander E. Felice in the Laboratory of Molecular Genetics with participation from many departments at the University of Malta. Dr. Joseph Borg and Mr. Clint Mizzi are involved in genome sequence analysis of a selection of the Maltese population. The team headed by Professor Christian A. Scerri of the Thalassaemia and Molecular Genetics Clinic at Mater Dei Hospital is involved in clinical diagnostic testing.

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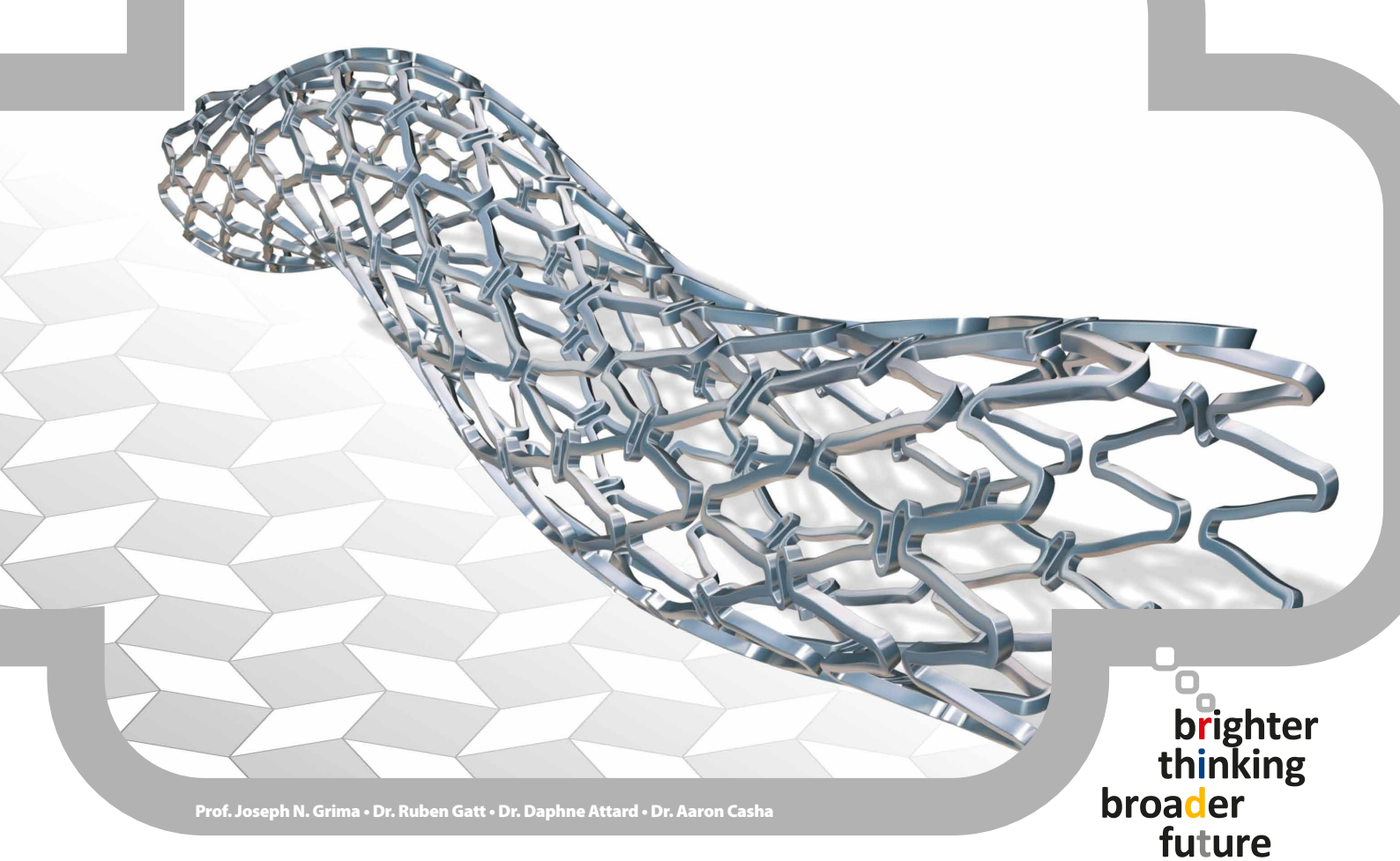

Prof. Joseph N. Grima • Dr. Ruben Gatt • Dr. Daphne Attard • Dr. Aaron Casha

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Metamaterials

The Research

Typically materials which we encounter in our everyday life get their properties as a result of their chemical composition alone. However, our quest for having new materials with previously unachievable properties has led to the development of a new class of materials, now referred to as 'metamaterials', i.e. engineered systems that exhibit macroscopic properties that emerge due to the structure of their subunits rather than their materials composition. Our team works with materials and metamaterials which get fatter when stretched (auxetics), shrink when heated or expand when under pressure.

How it Makes Our Lives Better

By designing tailor made materials having unusual properties, we can design superior products. For instance auxetics may be used in the design of highly efficient stents, cushions, filters, skin grafts, etc.

The Research Team

The metamaterials research team is lead by Professor Joseph N. Grima, Dr. Ruben Gatt and Dr. Daphne Attard from the Metamaterials Unit of the Faculty of Science in collaboration with Dr. Aaron Casha from the Faculty of Medicine and Surgery at the University of Malta.

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Dr. Pierre Schembri-Wismayer • Ms. Analisse Cassar

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Novel Approaches to Cancer Treatment

The Research

Healthy blood cells mature through a process called differentiation, a process by which cells divide and become more specialized in what they do. After a period of time normal cells stop dividing and die. Leukaemia is an example of a blood cancer where cells do not differentiate, and rather remain immature and immortal. Consequently, the cells keep dividing and growing faster than normal healthy bone marrow and blood cells eventually causing sickness and death. The team is focused on finding new molecules that will encourage leukaemia and cancer cells to differentiate and die off naturally. We have already discovered extracts from insects and plants that are successful in causing differentiation in a range of leukaemia cells. We collaborate with the European COST action STEMCHEM in screening more such compounds.

How it Makes Our Lives Better

Some cancers, including certain blood cancers called Acute Myeloid Leukaemia, are difficult to treat. The current treatment is chemotherapy, which is only partially effective as it kills the cancer cells, but also poisons normal healthy cells causing many side effects including hair loss, vomiting and bleeding. Finding therapies that could make cancer cells mature and die normally would help to treat leukaemia without many of these side effects.

The Research Team

This research is being carried out by a number of undergraduate and postgraduate students, following on the initial work of Ms. Analisse Cassar, under the supervision of Dr. Pierre Schembri-Wismayer from the Department of Anatomy in the Faculty of Medicine and Surgery.

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Prof. Ray Fabri • Dr. Albert Gatt • Prof. Gordon Pace • Mr. Michael Rosner

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Language and Communication Technologies

The Research

Language is the point of intersection between imagination, reasoning, knowledge and expression. It constitutes the essence of humanity and a building block of civilisation. Yet in an era dominated by increasing globalisation, mechanisation and exposure to information, we are confronted by problems whose root causes are the barriers imposed by language itself: human languages with which we are not familiar; machine languages we find unnatural, awkward and inexpressive; above all, the bombardment of information that overloads our capacities. These problems are being addressed by technology, and our research explores technological solutions to language processing, studying language itself and experimenting with intelligent computer systems that use language. The research area is highly interdisciplinary, lying on the frontier of linguistics, computer science and artificial intelligence.

Solutions being developed address many different aspects of these problems: low-cost high-quality machine-translation of human languages; dialogue systems for spoken and written interaction so that we can communicate with machines naturally; computational models of languages like Maltese that are needed for spelling and style correction; tools for understanding semi-formal documents like contracts; automated interpretation of complex databases in linguistic terms understandable to humans.

The Research Team

The research team consists of Mr. Michael Rosner of the Department of Intelligent Computer Systems, Professor Ray Fabri and Dr. Albert Gatt of the Institute of Linguistics and Professor Gordon Pace of the Department of Computer Science of the University of Malta.

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