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2003 – 2007
Editors: Joseph N. Grima and Richard Muscat
Xjenza Vol. 12 (2007)
Xjenza Vol. 11 (2006)
Xjenza Vol. 10 (2005)
Xjenza Vol. 9 (2004)
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1996 – 2002
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Associate Editors: *Martin Ebejer and Richard Muscat*
Xjenza Vol. 5 (2000)
Xjenza Vol. 4 Iss. 2 (1999)
Xjenza Vol. 4 Iss. 1 (1999)

Scope of Journal

Xjenza Online is the Science Journal of the Malta Chamber of Scientists and is published in an electronic format. Xjenza Online 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 printed issue of the journal was published in 1996 and the last (Vol. 12) in 2007. The publication of Xjenza was then ceased until 2013 when a new editorial board was formed with internationally recognised scientists, and Xjenza was relaunched as an online journal, 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 a launching platform into scientific publishing for 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 Science Journal of the Malta Chamber of Scientists and is published by the Chamber in electronic format on the website: https://www.xjenza.org/. Xjenza will consider manuscripts for publication on a wide variety of scientific topics in the following categories

1. Research Articles
2. Communications
3. Review Articles
4. Notes
5. Research Reports
6. Commentaries
7. News and Views
8. Invited Articles and Special Issues
9. Errata

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.

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.

Review Articles describe work of interest to the wide community of readers of Xjenza. They should provide an in-depth understanding of significant topics in the sciences and a critical discussion of the existing state of knowledge on a topic based on primary literature sources. 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 describe new 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.

Research Reports are extended reports describing research of interest to a wide scientific audience characteristic of Xjenza. Please contact the editor to discuss the suitability of topics for Research Reports.

Commentaries Upon Editor's invitation, commentaries discuss a paper published in a specific issue and should set the problems addressed by the paper in the wider context of the field. Proposals for Commentaries may be submitted; however, in this case authors should only send an outline of the proposed paper for initial consideration. The contents of the commentaries should follow the following set of rules: 3000 words maximum, title 20 words maximum, references 10 maximum (including the article discussed) and figures/tables 2 maximum.

News and Views The News section provides a space for articles up to three journal 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.

Invited Articles and Special Issues Xjenza regularly publishes Invited Articles and Special Issues that consist of articles written at the invitation of the Editor or another member of the editorial board.

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 short and consistent for clarity.

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Manuscripts should be sent according to the guidelines given hereafter to xjenza@mcs.org.mt.

Referees 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.

Conflict of Interest Authors are expected to disclose any commercial or other types of associations that may pose a conflict of interest in connection to 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.

Policy and Ethics The work presented in the submitted manuscript must have been carried out in compliance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/); 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 Author(s) must only submit work that has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that is not under consideration for publication elsewhere, that 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, 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

Xjenza accepts submissions in MS Word, Libre Office Writer and LaTeX, the latter being the preferred option. Anyone submitting in LaTeX should use the journal template, the latest version of which can be found at https://www.overleaf.com/latex/templates/xjenza-article/ktbfsjgqqcpw. All the necessary files to run the LaTeX document should be supplied together with the rendered PDF.

If a word processor is used the styling should be kept to a minimum. Bold face and italic fonts, as well as subscript and superscript text may be used as required by the context. Text should be in single-column format and the word processor options should not be used in order to justify text or hyphenate words. Alongside the native format of the word processer, a PDF file, generated by the word processor, must be provided. Furthermore, artwork should be in accordance with the artwork guidelines given below and must be submitted separately from the word processor file. Similarly, the bibliographic data of the cited material should be submitted separately as an Endnote (*.xml), Research Information Systems (*.ris), Zotero Library (zotero.splite) or a BibTeX (*.bib) file.

Article Structure

A manuscript for publication in Xjenza will typicall have the following components: Title page, Abstract, Keywords, Abbreviations, Introduction, Materials and Methods, Results, Discussion, Conclusions, Appendices and References.

The manuscript will be divided into clearly defined and numbered sections. Each numbered subsection should have a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text, i.e. refer to the subsection by the section number.

Title page

- The title should be concise yet informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- Author names and affiliations. Indicate the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript number immediately after each author's name and in front of the appropriate address. Provide the 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.
- Present/permanent address. If an author has changed the address since the work described, this can be indicated as a footnote to the author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

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.

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 consistently 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.

Materials 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. Do not 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. Anyone using LaTeX should use the package siunitx in all cases.

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 by a superscript number in the text and list the footnotes separately at the end of the article. Do not include footnotes in the Reference list.

Table Footnotes Indicate each footnote in a table with a superscript lower case letter.

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- Make sure you use uniform lettering and sizing of your original artwork.
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Tables 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 moderate with the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article. Large tables should be submitted in CSV format.

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Citation in text References to cited literature in the text should be given in the form of an author's surname and the year of publication of the paper with the addition of a letter for references to several publications of the author in the same year. For further information regarding multiple authors consult the APA v6 guidelines. Citations may be made directly

Kramer et al. (2010) have recently shown . . .

or parenthetically

as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999).

Groups of references should be listed first alphabetically, then chronologically. When writing in LaTeX use `\textcite{}` and `\parencite{}` for the respective cases mentioned.

The reference section Every reference cited in the text should also be present in the reference list (and vice versa). The reference list should also be supplied as an Endnote (*.xml), Research Information Systems (*.ris), Zotero Library (zotero.splite) or a BiBTeX (*.bib) file. 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.

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. Consult the APA v6 guidelines for multiple authors. Below are some examples of referencing different bibliographic material.

Reference to a Journal Publication

Agree, E. M. and Freedman, V. A. (2011). A Quality-of-Life Scale for Assistive Technology: Results of a Pilot Study of Aging and Technology. *Phys. Ther.*, 91(12):1780–1788.

McCreadie, C. and Tinker, A. (2005). The acceptability of assistive technology to older people. *Ageing Soc.*, 25(1):91–110.

Reference to a Book

Brownsell, B. (2003). *Assistive Technology and Telecare: Forging Solutions for Independent Living*. Policy Press, Bristol.

Fisk, M. J. (2003). *Social Alarms to Telecare: Older People's Services in Transition*. Policy Press, Bristol, 1st edition.

Reference to a Chapter in an Edited Book

Brownsell, S. and Bradley, D. (2003). New Generations of Telecare Equipment. In *Assist. Technol. Telecare Forg. Solut. Indep. Living*, pages 39–50.

Web references The full URL should be given together with 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.

Journal Abbreviations Journal names should be abbreviated according to:

-Index Medicus journal abbreviations: https://www.ncbi.nlm.nih.gov/nlmcatalog/journals/;

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

- 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:
  - All figures are given separately in PDF, SVG, JPEG of PNG format.
  - Caption for figures is included at the end of the text.

  - All tables (including title, description, footnotes) are included in the text and large tables have been given separately as CSV.
  - The reference list has been given in XML, RIS, zotero.splite or BIB file format.
- Further considerations
  - Abstract does not exceed about 250 words.
  - 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.
  - Bibliographic data for all cited material has been provided.
  - Permission has been obtained for use of copyrighted material from other sources (including the Web).
  - A PDF document generated from the word processor used is submitted.

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Editorial

Top Research In Malta

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Dear Xjenza Online Readers,

Following the success of the inaugural Special Issue on Top Research in Malta, we have decided to launch a new collection featuring researchers who were among the top 2% of the most cited authors globally in 2021. We were pleasantly surprised to see the inclusion of young colleagues alongside the distinguished scholars from the previous edition. The 2021 top 2% list was published by Stanford University and Elsevier and is available at https://elsevier.digitalcommonsdata.com/datasets/btchxktzyw/4.

In fact, in 2021, we had twenty scientists based in Malta – twelve of whom are affiliated with the University of Malta.

This list of the most widely cited researchers in their fields was compiled by Prof. John Ioannidis and his team at Stanford University. They analyzed data from 1965 to 2020, covering around 7 million scientists in 22 major fields. The list identifies the top 100, 000 scientists across all fields and was published in PLOS Biology in 2022 (Ioannidis, 2022).

To provide a broader snapshot of the best science in Malta, we invited not only the 22 researchers included in Ioannidis' list but also those listed among the top ones in Google Scholar User Profile rankings when you search for the University of Malta (Google Scholar, 2024) , where scholars are listed according to the number of their citations.

Readers will find 11 invited articles in this second part of the Special Issue, with others scheduled for future editions on Top Research in Malta.

In the wake of the COVID-19 pandemic, non-communicable diseases (NCDs) have emerged as pressing global concerns. The issue begins with Dr. Sarah Cuschieri's article which highlights the impact of COVID-19 on NCDs, intensifying the burden on healthcare systems and exacerbating individuals' well-being. With prevalent conditions like type 2 diabetes, obesity, and low back pain in Malta, the pandemic has spotlighted these issues alongside mental illness and obesity. Despite challenges, vaccination rollouts offer relief in dampening the NCD burden. However, Dr. Cuschieri emphasizes the need for a syndemic approach to address intersecting epidemics, safeguarding population health and well-being through holistic strategies.

The issue continues with a contribution by David Pace on Clinical vaccine research in children in Malta. Clinical vaccine trials in children have become pivotal endeavors in pediatric healthcare, offering insights into optimizing vaccination schedules for existing vaccines. Dr. David Pace, in collaboration with esteemed colleagues in the UK, conducted significant clinical research on meningococcal C vaccines in children from 2010 to 2013. Their work, detailed in peer-reviewed journals, offers valuable insights into reduced-dose schedules and antibody response kinetics post-booster doses in infants. Such endeavors hold immense practical significance, particularly in informing national immunization policies, evident in the introduction of meningococcal vaccines into Malta's schedule. It's imperative that Malta's medical community continues to champion pediatric research, supported by clinical and academic institutions, to advance pediatric healthcare.

Advancements in electrical power systems are discussed by Alexander Micallef from the University of Malta's Faculty of Engineering. Insights into the microgrids research team's recent endeavors within the Department of Industrial Electrical Power Conversion (IEPC) are provided. Their work aims to foster secure, reliable, and environmentally sustainable electricity systems, focusing on enhancing microgrid operation, control, and management. Significant achievements include hierarchical control architecture development, innovative control algorithms, energy management strategies, power quality enhancement technologies, demand response techniques, and renewable energy source integration. These advancements

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Baldacchino, Godfrey	University of Malta
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Grima, Joseph N.	University of Malta
Grech, Victor	Mater Dei Hospital
Scarpignato, Carmelo	United Campus of Malta
Borg, Michael A.	Mater Dei Hospital
Yannakakis, Georgios N.	University of Malta
Balzan, Mario V.	Malta College of Arts, Science & Technology
Valdramidis, Vasilis	University of Malta
Gatt, Ruben	University of Malta
Pace, David	Mater Dei Hospital
Sultana, Janet	Mater Dei Hospital
Attard, Daphne	University of Malta
Makantasis, Konstantinos	University of Malta
Magri, David C.	University of Malta
Francalanza, Adrian	University of Malta
Di Giovanni, Giuseppe	University of Malta

Table 1: The twenty Maltese scholars, as found in the 2021 top 2% list published by Stanford University and Elsevier.

underscore the department's commitment to shaping the future of electricity distribution through microgrid technology.

The latest research by Mark Anthony Camilleri and Adriana Caterina Camilleri highlights the importance of inclusive education, lifelong learning, and active labor market policies for societal well-being. Their comparative analysis of socio-economic policies in Malta and Cyprus underscores the need to attract more students to vocational and higher education to enhance employment prospects. Focused on vulnerable groups, their findings emphasize targeted labor market policies to support those not engaged in employment, education, or training. Continuous improvements in education quality and social cohesion are vital for positive outcomes like job creation and societal well-being, indicating the significance of effective policies for economic growth and social progress in Mediterranean island states.

Jean Claude Scicluna and Giuseppe Di Giovanni's systematic review addresses the pressing issue of fibromyalgia, a condition associated with significant morbidity and economic burden. Despite minimal benefits from traditional pharmacotherapies, there's growing interest in novel treatments. The endocannabinoid system has emerged as a potential target, yet the therapeutic potential and adverse effects of cannabis-based therapy remain underexplored, leading to clinician hesitancy. This review critically evaluates the safety and efficacy of cannabis-based therapy for fibromyalgia, concluding that it presents a safe and effective treatment option. However, further research is warranted to fully understand its potential in managing this debilitating condition.

Gianluca Valentino and colleagues present a comprehensive overview of Interferometric SAR (InSAR) phase denoising and phase unwrapping techniques in their paper. These two critical steps in the InSAR pipeline are essential for generating accurate deformation maps. The paper surveys recent literature in this field, highlighting promising techniques and establishing benchmarks for performance evaluation. Additionally, the authors provide summaries of performance metrics for various methods. To illustrate the practical application of InSAR techniques, they offer an example of estimating deformation following a volcanic eruption. This study serves as a valuable resource for researchers and practitioners in the field of SAR image processing.

Georgios N. Yannakakis delves into the symbiotic relationship between artificial intelligence (AI) and digital games, highlighting how advancements in AI have revolutionized both fields. He explores how AI algorithms have driven breakthroughs in machine learning, search, and optimization, directly impacting game design and complexity. Yannakakis emphasizes the pivotal role of games in AI research and the reciprocal influence of AI on game

development. In the second part of the paper, he focuses on the Institute of Digital Games at the University of Malta as a prominent center for AI and games research, education, and innovation. Through targeted investment and national focus, Malta has emerged as a global leader in AI and video game development within a remarkably short timeframe, showcasing the transformative potential of strategic investment in this domain.

Brendon Scicluna explores the challenges of sepsis due to its heterogeneous nature, hindering the identification of effective treatments. He reviews current efforts in precision medicine, aiming to stratify sepsis patients into more uniform subgroups for targeted therapeutic interventions, ultimately enhancing treatment outcomes.

In their study, Balzan Mario and Leticia De Santis explore the influence of landscape and local habitat variables on honeybee and wild bee populations in the Maltese Islands. They find that honeybees, associated with agricultural habitats, overlap in resource use with wild bees. While different habitats support diverse bee groups, landscape factors like arable land and grassland positively affect wild bee abundance. High honeybee visitation negatively impacts wild bee abundance but does not significantly affect functional group richness. The study emphasizes the need for holistic habitat management approaches to conserve bee diversity and pollination services in the region.

David Magri's mini-review delves into the significance of the 2022 Nobel Prize in Chemistry, honoring the breakthroughs in click chemistry and biorthogonal chemistry. He focuses on two innovations: the Pourbaix sensor and the Lab-on-a-Molecule. These molecules, designed with fluorescence properties, operate through a balance between non-radiative photoinduced electron transfer (PET) and radiative fluorescence, modulated by various equilibrium states. Magri highlights their potential applications in corrosion detection, cell imaging, and health diagnostics, emphasizing their societal benefits.

The collection ends with the contribution of Alex Felice, one of the founders of Malta Chamber of Scientists, intertwining two narratives that deeply resonate with his professional journey. The first narrative is personal, tracing his career path from Malta to the USA and back, influenced by family and mentors, as well as the many graduate students and trainees he has mentored. This personal journey seamlessly merges with the second narrative, which focuses on the contributions made by Felice and his team to the study of Human Haemoglobinopathy. His research has shed light on the quantitative effects of genetic co-regulators on complex phenotypes, revealing how mutations at multiple alleles across different loci can influence the expression of βglobin gene variants. This intricate interaction extends to other molecules like α-globin and KLF1, the master regulator of Erythropoiesis, offering insights into rare diseases. Felice also highlights the impact of their work in training new health and academic professionals, establishing new resources and laboratories, and initiating international collaborations, all of which hold promise for expedited diagnoses and novel treatments in the field of rare diseases.

I would like to thank all the contributors who have made this volume exceptional, showcasing 11 of the most important acclaimed UM academics. I am grateful to these authors for their high standard of work and to the reviewers for their crucial help in the peer-review process.

I look forward to further issues of this Special Issue of Xjenza Online on Top Research in Malta series and wish all the best to all Maltese researchers.

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Review Article

Living in the Era of Multiple Epidemics—A Malta Perspective

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Abstract. Non-communicable diseases (NCDs) have long been a global epidemic way before the onset of the COVID-19 pandemic. In 2016, it was estimated that 6.55% of the adult population in Malta suffered from at least one NCD, with type 2 diabetes, obesity and low back pain dominating the NCD scene. The onset of COVID-19 challenged the healthcare systems, as well as the wellbeing of the population. Restrictions instituted to control COVID-19 led to negative repercussions on those suffering from NCDs apart from bringing to the fore specific NCDs such as mental illness, obesity, and back pain. Furthermore COVID-19 increased the population burden through enhanced morbidity and mortality. However, the COVID-19 vaccination was observed to have helped dampen this burden. Yet, it is important that a syndemic approach is adopted to ensure that all epidemics are simultaneously given the appropriate attention and timely action is provided to safeguard the population health and wellbeing.

Keywords: Non-communicable diseases, Epidemics, COVID-19, Healthcare, Population Health Malta

1 Introduction

1.1

Non-communicable diseases (NCDs) have long been acknowledged as being a global epidemic contributing to a substantial population burden, with enhanced morbidity and premature mortality (Vos et al., 2020). These have been dominating the world way before the onset of the SARS-CoV2 coronavirus epidemic in 2019 (Peng et al., 2020). Non-communicable diseases, also referred as chronic diseases, are non-infectious diseases that have a long-term impact on the effected population's health and wellbeing. Indeed, in 2019, it was estimated that NCDs contributed to 91.08% (4.9 million) deaths and 87.55% of all Disability-Adjusted Life Years (DALYs) across the countries making up the European Union (EU)(Institute for Health Metrics and Evaluation, 2020).

The most commonly reported NCDs are cardiovascular diseases, chronic respiratory diseases, diabetes and cancers (World Health Orgainzation, 2018). These NCDs have been linked to common behavioural risk characteristics namely poor diet, tobacco use, excessive alcohol consumption and lack of physical activity (Hunter et al., 2013; World Health Orgainzation, 2016a). Poor diet refers to the consumption of the extensive availability and highly affordable processed food which is high in salt, sugar, fat, and calories. The consumption of which, leads to an enhanced body weight and waist circumference. Both of which are risk factors for the development of several NCDs (Webber et al., 2014; World Health Orgainzation, 1994). The digital era we are living in, is further challenging the NCD epidemic as food delivery applications have been linked with increased consumption of unhealthy diet. Consequently increases the risk of NCD development (Halloran et al., 2022).

Yet, most NCDs can be preventable by tackling the risk factors such as unhealthy diet, tobacco and alcohol use, sedentary lifestyle as well as environmental factors. Indeed, 80% of diabetes heart disease and stroke cases can be prevented, as can 40% of cancer cases (World Health Orgainzation, 2020a). Additionally, NCDs is an economic burden at the individual level, on the healthcare delivery and at a national level (Hofmarcher et al., 2020). In fact, the EU Member States account for at least 25% of their national healthcare spending on the four commonest NCDs (cardiovascular disease, diabetes mellitus, chronic respiratory disease, and cancer). Furthermore, these four NCDs also account for 2% economic loss from the EU Member states gross domestic product (GDP) (Hofmarcher et al., 2020). Countries have set up several national strategies and policies addressing the NCD epidemic, yet most countries lack the capacity to implement the interventions, subsequently resulting in a worse NCD

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state (Breda et al., 2019).

1.2 NCDs situation in Malta pre-COVID-19

The presence of the NCD epidemic is evident in the small Mediterranean Island of Malta, with type 2 diabetes being referred to as the national disease (Cuschieri et al., 2014). Indeed, the last nationwide cross-sectional health examination survey conducted (2016) in Malta, estimated that 10.31% of the adult population suffered from type 2 diabetes. 4% out of these were not aware of having this disease before taking part in this study (Cuschieri, 2020c). Another pressing epidemic in Malta, mirroring the global landscape, is obesity (Cuschieri, 2020a; World Health Orgainzation, 2014). In 2016, 69.75% of the adult population in Malta was identified to be either overweight (body mass index (BMI) >25 Kg/m^2 but <30 Kg/m^2 or obese (BMI >30 Kg/m^2) (Cuschieri et al., 2016a). Meanwhile on a global level, in 2016 it was reported that 39% of adults were overweight and 13% were obese (World Health Orgainzation, 2016b). This high occurrence of obesity in Malta has been attributed to the obesogenic environment the population lives in, as well as being associated with the male sex, increase in age, suffering from diabetes or impaired fasting glucose (IFG) (Cauchi et al., 2015; Cuschieri, 2020a). Another factor contributing to both the diabetes and obesity epidemics is the transition in the nutritional process from the traditional Mediterranean diet to a Westernized diet. This shift is being experienced across all the Mediterranean countries including in Malta (Cuschieri et al., 2021h; Da Silva et al., 2009; Dinu et al., 2021). The high prevalence of diabetes and obesity contributes to a high population burden, not only at an individual level but also at a national level, with increased economical and health service burdens. Indeed, it was estimated that the economic burden for both diabetes and obesity for the year 2016 was €52,891,997 equivalent to 6.61% of the total health expenditure for Malta (Cuschieri et al., 2016b).

Chronic low back pain is another non-communicable disease and reported to be a major health problem in Europe, with a prevalence of 19% among the adults in Europe (Breivik et al., 2006). In Malta, in 2015 it was estimated that 6.4% of the adults suffered from chronic low back pain, which contributed to 2,633 healthy life years lost in a year (716 disability adjusted life years DALYs per 100,000) (Cuschieri et al., 2020d; Directorate for Health Information and Research, 2018).

In the current ageing era, it is more common for the population to suffer from two or more concurrent NCDs, also referred to as multimorbidity, which is a pressing public health concern (Garin et al., 2016; Li et al., 2016). In 2016, 33% of the adults in Malta suffered from multimorbidity, with the commonest concurrent diseases being myocardial infarction and hypertension (54.45%) (Cuschieri et al., 2021d). Interestingly, Gozo, was observed to have the highest multimorbidity prevalence when compare to the main island (Cuschieri et al., 2021d).

1.3 The Clash of Epidemics

The end of the year 2019 saw the emergence of the novel coronavirus SARS-CoV2, causing the COVID-19 infection. Within a short period of time, COVID-19 became an epidemic and by March 2020 it was declared a pandemic (World Health Orgainzation, 2020b). This came at the backdrop of the soaring NCDs epidemic that was contributing to a large global burden, disability and overloading healthcare services (GBD 2019 Deseases and Injuries Collaborators et al., 2020). During the first phase of the COVID-19 pandemic, most governments, including in Malta, instituted a number of restrictions including the postponements of elective surgeries and consultations to prioritize health resources towards COVID-19 management, along with mandates for social distancing and lockdowns (Cuschieri, 2020b; Tabari et al., 2020). These restrictions also had a negative effect on the screening and management of NCDs, with an anticipated negative effect in the long term (Palmer et al., 2020). Indeed, in the United Kingdom between March and December 2020 it was an estimated that 60,000 individuals with type 2 diabetes were missed (Carr et al., 2021). Although these measures enabled the curbing of the viral spread, they imposed additional challenges for those already suffering from NCDs apart from increasing health inequalities (Cuschieri et al., 2021i). It needs to be noted that those suffering from NCDs, including diabetes and obesity, exhibited a higher susceptibility to get complications if infected by COVID-19 (Cuschieri et al., 2020b, 2020c; O'Donovan et al., 2019). Therefore, the collusion of these two epidemics increased the burden of COVID-19 through morbidity and mortality (Azarpazhooh et al., 2020). Hence protecting the vulnerable, including those suffering from NCDs was the way forward before vaccination came in play (Cuschieri et al., 2021g). Additionally, the pandemic brought about an increased mental health burden even among the previously healthy population (Pan et al., 2021). The fear and anxiety brought about by COVID-19 also impacted negatively on the willingness to seek medical care especially among those already suffering from NCDs, as was observed in a Malta Survey (Cuschieri et al., 2021f).

1.4 The Impact of COVID-19 in Malta

The first reported COVID-19 case in Malta was on the 7th of March 2020 (Cuschieri, 2020b). Timely measures, adequate hospital preparedness along with the population's

cooperation led to a low burden on the healthcare system as well as minimal population morbidity and mortality during the first wave (Cuschieri, 2020b; Cuschieri et al., 2021c). Indeed, compared with other small islands in Europe, Malta had the best first wave COVID-19 outcome (Cuschieri et al., 2021j). Gradual lifting of restrictions in May 2020 saw Malta move into the transmission period and enter Summer 2020 (Cuschieri, 2021). However, unrestricted mass events tipped the scales and Malta entered into a rapid incline in COVID-19 cases and mortality as of August 2020 (Cuschieri et al., 2020a). Over the first year of COVID-19, it was estimated that the direct burden of COVID-19 imposed on the Maltese population contributed to 5,478 (DALYs) healthy life years lost, with the highest contributing factor being mortality (Cuschieri et al., 2021b). It was noted that the burden of COVID-19 ranked the fourth leading disease in Malta following ischemic heart disease (1^{st}), low back pain (2^{nd}) and diabetes (3^{rd}) (Cuschieri et al., 2021b). The end of 2020 saw the approval of the first COVID-19 vaccine and the start of the vaccination programme across Europe (European Medical Agency, 2021). Malta was able to secure enough vaccine doses for the whole eligible population and adopt a rapid vaccination strategy to inoculate the population over a couple of months (Cuschieri et al., 2021a). It was expected that the COVID-19 burden on healthcare systems and on the population reduced as the vaccination update increase. Indeed, the positivity rate (number of positive cases divided by the number of swabs) and the mortality rate among the 65+ years decreased drastically only a few months following the vaccination uptake (Cuschieri et al., 2021g).

1.5 Covid-19 Impact on NCDs

Taking Malta as a case study, it was estimated that 6.55% of the adult population suffers from at least one NCD (Cuschieri et al., 2021e). These are known to be more susceptible to acquire COVID-19 complications and may need medical attention with a possibility of hospital admission. A predictive modeling exercise was carried out, before the initiation of the COVID-19 vaccination programme, using local data (Malta). This exercise estimated that 20,527 Maltese adults suffering from NCDs were at risk of requiring hospitalization if infected by COVID-19 (Cuschieri et al., 2021e). Of note, those suffering from NCDs are associated with having a higher risk of developing depressive illness that inevitably will have a negative repercussion on the individual's health outcome (Chudasama et al., 2020). Additionally, individuals suffering from NCDs that survive the COVID-19 infection are at higher risk of enhancing the progression of their pre-existing NCDs as well as develop long-haul symptoms (Al-Jahdhami et al., 2021; Palmer et al., 2020).
The COVID-19 restrictions brought additional risks towards developing new onset NCDs. A survey conducted among Maltese adults observed that 49% of the responders started to experience back pain following the onset of COVID-19. Indeed, the factors associated with back pain were identified to be prolonged sitting down (15% increase risk) and lack of physical activity (4% increase risk) (Grech et al., 2022). Prolonged periods at home and restricted access to leisure areas tend to increase the sedentary behaviour and screen time, all of which are associated with higher risk of weight gain even among children (Tripathi et al., 2020). Increased screen time is also associated with increased snacking frequency, which further enhances the risk of weight gain (Tripathi et al., 2020). Additionally, the COVID-19 pandemic is a psychological stress on its own, which may result in stress-eating of high calorie food and drinks, which also increases the risk of weight gain (Pietrobelli et al., 2020). Therefore, development of obesity is an indirect effect of COVID-19 (Jia et al., 2021; Mulugeta et al., 2021).

2 Implications to Policy and Practice

As the COVID-19 pandemic keeps on soaring across the world, containment, curbing the spread and protecting the population remains on top of the policy agenda. Yet, it is important to remember the NCDs epidemic has been a global emergency way before the onset of the COVID-19 pandemic. While controlling the viral spread will indirectly reduce the progression of NCDs and the development of pandemic related new onset NCDs, direct action targeting the NCD epidemic is required (Cuschieri et al., 2021d; Cuschieri et al., 2021i). Indeed, a syndemic approach has been proposed, where both COVID-19 and NCDs are managed simultaneously. The primary healthcare should be the hub for this management as it offers holistic care as well as being located within the community, enabling a better access to medical care. Furthermore, shifting such care to the primary healthcare will reduce the burden on the tertiary healthcare systems which will enable these systems to dedicate their resources to treating the acute and critically ill population (Cuschieri et al., 2021i).

3 Conclusion

We are living in an era of multiple concurrent epidemics, some of which have conquered the world for decades. Malta, like the rest of the world, has been in the constant battle to control the growing epidemic of NCDs. The onset of the COVID-19 pandemic shifted the focus temporarily from the NCD epidemic, causing the disruption and potentially negative repercussions to the long-term management of NCDs. It is therefore important that

a syndemic approach is adopted to ensure that all epidemics are simultaneously given the appropriate attention and timely action is provided to safeguard the population health and wellbeing.

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Research Article

Clinical vaccine research on Meningococcal C disease in children in Malta

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Abstract. Clinical vaccine trials in children are extremely important for the investigation of new vaccines as well as for studying different ways of scheduling vaccines that are currently in use. Data from such trials, in addition to epidemiological data on the infectious disease the vaccines are trying to prevent, can be used to introduce vaccines as well as to improve the current immunisation schedules. The purpose of this review is to showcase the clinical vaccine research on meningococcal C vaccines in children that was carried out in Malta in collaboration with the UK from 2010 to 2013, data from which have already been presented and published in peer reviewed journals. This review gives a synopsis of the immunogenicity of reduced dose meningococcal C vaccine schedules in infants as well as the immune kinetics of the antibodies induced following a booster dose at 12 months of age. The practicality of the study findings are discussed, including their relevance to the meningococcal vaccines that were recently introduced on the national immunisation schedule in Malta. Hopefully this research will encourage doctors to show interest in leading future research in children in Malta with appropriate support from our clinical and academic institutions.

Keywords: Clinical Research, RCT, Meningococcal C Vaccines, Paediatrics

1 Introduction

Qualifying and practising as a medical doctor starts to bring up lots of why, what, who, when, where and how questions. Some of these may be addressed by browsing through and critically analysing the medical literature but others may remain unanswered. These unanswered questions lead to the creation of ideas and the formulation of hypotheses that are addressed through research methods. Such questions become even more important when working in Paediatrics. The inherent vulnerability of children and infants provide a challenge to the conduction of clinical trials in children, although Good Clinical Practice (GCP) guidelines which set ethical and scientific standards in research and which are implemented by the EU Clinical trial directive (European Medicines Agency) have made this more feasible. Caring for children with infections, especially those with meningitis and septicaemia that may be fatal or potentially disabling, makes one wonder: considering all the advances in science and technology are we doing enough to control such infections effectively in the 21st century? Clinical practice and research are complimentary in Paediatric Infectious Diseases and provide a holistic approach to children suffering from infections. The importance of research to clinical practice has become ever more recognised during the current SARS-CoV-2 pandemic (World Health Organization, 2020).

One of the major pathogens causing meningitis and septicaemia globally is *Neisseria meningitidis* which mainly affects infants, children below four years of age and adolescents (Centers for Desease Control and Prevention, 2021; European Cetre for Disease Prevention and Control, 2019). Besides endemic disease the meningococcus has the potential to cause epidemics during which older children and adults are also affected (Tyrrell et al., 2002). Despite the rarity of invasive meningococcal disease (IMD) when compared with other childhood infections, such as lower respiratory tract infections and gastroenteritis (World Health Organization, 2021), the meningococcus remains a major public health concern due to the rapidity of disease progression, its potential for causing outbreaks and the associated permanent disabling sequelae that may occur from a very young age.

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2 Epidemiology of meningococcal disease

The meningococcus is classified into 12 different capsular groups based on the biochemical composition of the polysaccharide capsule, with groups A, B, C, W, Y, and more recently X (Delrieu et al., 2011), being responsible for 90% of the global meningococcal disease burden. Capsular groups B and C are the most prevalent groups in Europe and in the US, where capsular group Y is an equally important cause of IMD (Centers for Desease Control and Prevention, 2021; European Cetre for Disease Prevention and Control, 2019). Since the 1950s, the overall mortality from IMD has remained around 8-10% despite advances in intensive care and prompt initiation of appropriate antibiotics (Centers for Desease Control and Prevention, 2021; European Cetre for Disease Prevention and Control, 2019; Sadarangani et al., 2015). Between 7-20% of survivors aged up to 18 years suffer permanent disabilities, including hearing loss, seizures, neurodevelopmental impairment and amputations (Davis et al., 2011; Stein-Zamir et al., 2014). Capsular group C meningococcal disease is associated with a mortality rate of 11-15%, which is higher than the 6-10% case fatality rate for MenB disease (Cohn et al., 2010; Sadarangani et al., 2015; Xu et al., 2012), and with a 10-20% risk of permanent neurodevelopmental and/or physical disabilities (Stoof et al., 2015; Wang et al., 2014). Virulence more likely reflects genomic rather than capsular differences between different strains, with sequence type (ST) 11 meningococci, classically associated with the group C capsule, still behaving more aggressively than other strains even when expressing a different capsular group (Ladhani et al., 2015). A rational and cost-effective strategy for preventing capsular group C disease is through routine childhood vaccination programmes (De Wals et al., 2004; de Soarez et al., 2011; Trotter et al., 2006; Welte et al., 2004).

3 Meningococcal C vaccines

A rise in MenC disease caused by a hyperinvasive ST11 clone led to the development of glycoconjugate MenC vaccines in the 1990s. Glycoconjugate vaccines consist of an oligo/polysaccharide extracted from the capsule of a bacterium which is chemically conjugated to a protein, known as the carrier protein. Three MenC glycoconjugate vaccines were formulated; two having the cross reactive material (CRM197), a non-toxic mutant of diphtheria toxoid, as a carrier protein (Menjugate, GlaxoSmithKline Vaccines, Siena, Italy and Meningitec, withdrawn but previously produced by Nuron Biotech, Schaffhausen, Switzerland) and one utilising tetanus toxoid (NeisVac-C; Pfizer Inc., New York, US). Control of MenC disease has been largely achieved with the introduction of these glycoconjugate vaccines on national immunisation schedules within Europe, with the UK being the first to introduce MenC conjugate vaccination back in 1999 for routine vaccination of infants with a concurrent one time catch-up vaccination of 1-25 year olds (Campbell et al., 2009). The success of these MenC conjugate vaccination programmes was not only a result of direct protection induced by vaccinating infants and toddlers but also a result of decreased transmission induced by catch-up vaccination of adolescents and young adults who are known to have high meningococcal carriage rates reaching up to 25% in 15-19-year-olds (Cartwright et al., 1987) and 32% at 25 years of age (Claus et al., 2005).

Since 1999, the MenC vaccination schedule in the UK was changed three times. The 2, 3 and 4 month MenC infant vaccine priming schedule (priming refers to the initial immune response observed after the first vaccination course) was reduced to a 3 and 4 months schedule with the introduction of a MenC booster dose, incorporated within a combined Haemophilus influenzae type b and MenC tetanus toxoid conjugate vaccine, Hib-MenC-TT, at 12 months of age in 2006 (Campbell et al., 2009). This change followed demonstration of robust immunogenicity with two infant priming MenC conjugate vaccine doses (Richmond et al., 1999) and waning of vaccine effectiveness by 12 months of age (Trotter et al., 2004). Subsequently, the almost complete disappearance of MenC disease in infancy led to reduction of infant priming to a single dose at 3 months of age in 2013 (Public Health England) but with the concurrent introduction of an adolescent boost at 13-14 years of age to maintain adolescent immunity and prevent meningococcal transmission to infants (Pollard et al., 2013). Thereafter the infant MenC dose was removed completely in 2016 since the extremely low rates of infant MenC disease were sustained, although the adolescent MenC dose, as part of the MenACWY conjugate vaccine that had replaced the monovalent MenC conjugate vaccine in 2015 due to an outbreak caused by MenW disease, was retained to maintain herd immunity (Public Health England, 2016).

Reducing the number of infant vaccine doses makes infant vaccination schedules easier to manage due to the ever increasing vaccines that are recommended in this age group. Vaccine schedules with less injections help to increase vaccine uptake by being more attractive to parents. The science behind a change in immunisation schedules comes from clinical vaccine trials designed specifically to address the immunogenicity of reduced dose schedules in conjunction with surveillance of the infectious disease that the vaccines are aimed to prevent. This article will showcase research looking at the prevention of meningococcal

C disease in children that was conducted in Malta and the UK. Data from this research have already been published in peer reviewed journals (Pace et al., 2016; Pace et al., 2015). Dissemination of the findings, which is a result of lots of hard work and long hours invested in conducting the research and which ultimately may have an impact on current practice, is the ultimate goal of any researcher.

4 Clinical research in Malta: A Clinical Vaccine trial

4.1 Study Design

The immune response to reduced dose MenC vaccine schedules was investigated in a Phase IV open label randomised controlled vaccine trial conducted in four sites in the UK, namely Oxford, Bristol, London and Southampton and in one site in Malta (Pace et al., 2015). A clinical vaccine trial site was set up at Mater Dei Hospital, Malta in collaboration with the Oxford Vaccine Group at the University of Oxford. Approvals were obtained from the respective research ethics committees and medicinal regulatory agencies in each country (UK NRES REC No: 10/H0604/7 and Malta HEC No: 24/10). At the time that this study was performed the UK was using 2 infant doses of a MenC conjugate vaccine at 2 and 4 months of age together with a booster dose, as part of the Hib-MenC-TT conjugate vaccine at 12 months of age.

In brief, 509 healthy infants were enrolled when aged between 6-12 weeks and randomised in a 10:10:7:4 ratio into 4 groups as follows: a single infant dose MenC-CRM197 group, a two infant dose MenC-CRM197 group; a single infant dose MenC-TT group and a control group reflecting the number of doses and the formulation of MenC conjugate vaccines given in infancy. The MenC-CRM197 conjugate vaccine formulation was Menjugate (GlaxoSmithKline Vaccines, Siena, Italy) and was given at age 3 months or at 3 and 4 months in the single and two infant dose MenC-CRM197 groups, respectively. NeisVac-C (Pfizer Inc., New York, US) was the MenC-TT formulation given at 3 months of age in the single infant dose MenC-TT group whilst infants in the control group did not receive any MenC vaccine doses in infancy. Following this primary vaccination phase participants proceeded to the booster phase in which the Hib-MenC-TT vaccine (Menitorix, GlaxoSmithKline Biologicals, Rixensart, Belgium) was given at 12-13 months of age. Antibodies against MenC were followed up until 24 months of age. All participants received the other routine vaccinations according to the immunisation schedule in the UK. In the booster phase participants in all groups were vaccinated with the Hib-MenC-TT vaccine at 12 months of age. Blood samples were obtained at 5, 12, 13 and 24 months of age. A subgroup of 64 participants randomly selected from each of the groups had a blood sample six days after the 12 month Hib-MenC-TT vaccine. Following each immunisation participants were observed for 15 minutes for any anaphylactic reactions and parents documented any local or systemic side effects (adverse events) for 5 days later. A MenC serum bactericidal antibody assay, which measures functional antibody, against N.meningitidis C11 (C:16:P1.7-1,1) strain, using baby rabbit complement (MenC-rSBA) was used to measure antibodies against MenC (Pel-Freeze Incorporated, Rodgerson, AZ). In order to assess any statistical significant difference between the standard two dose MenC vaccine schedule and the reduced single dose MenC schedule being studied, a primary objective was set to demonstrate non-inferiority in the MenC rSBA geometric mean titres (GMTs) one month after the 12 month Hib-MenC-TT vaccine. Non-inferiority was met if the lower 95% CI of the difference in the mean log10 MenC rSBA between the single minus the two infant dose MenC-CRM197 groups was >-0.35 (equivalent to a non-inferiority margin of >-10%), An analysis of variance (ANOVA) of the log10 transformed rSBA titres was performed at each blood sampling visit and results presented as GMTs with 95% CIs. A regression model was used to analyse the immune kinetics between the blood sampling visits, including the pre-boost, 6 and 28-day post boost antibody titres (Pace et al., 2016). The aim was to detect a 10% difference between those primed with any MenC conjugate vaccine compared to the unprimed control group. The GMTs, Geometric Mean Fold Rise (GMFR) and Geometric Mean Ratios (GMR) and their 95% Confidence Intervals (CI) were calculated to assess differences in the between the pre-boost and 6 and 28th day post boost antibody titres. For analysis of safety a logistic regression was used to assess binary variables and odds ratios with 95% CI were obtained when comparing two levels of a factor. P values less than 0.05 were considered statistically significant. Immunogenicity analysis was performed using STATA 13 and StatXact 9 whilst SAS v9.3 was used for analysis of safety.

4.2 Results

A total of 509 subjects were recruited with a mean age of 8.5 weeks (Range: 6.9 – 10.6) at enrolment. Gender ratio was balanced, with 51.7% (263) being males and 90.2% were Caucasian.

Following the Hib-MenC-TT boost at 12 months of age participants in the single infant MenC-CRM197 group had MenC rSBA GMTs of 660 [95% CI: 498 to 876] compared to 295 [95% CI: 220 to 398]) in the two infant dose MenC-CRM197 group (figure 1). The difference in

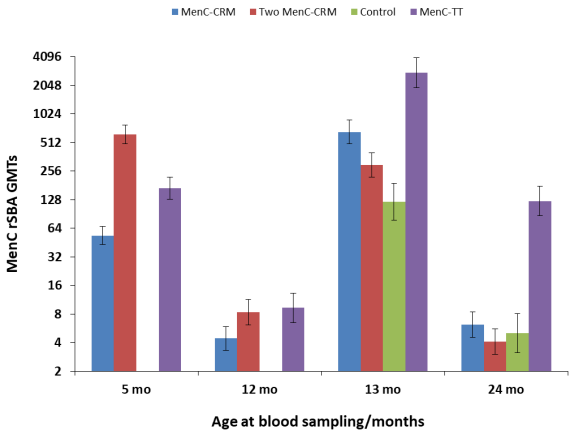

Figure 1: MenC rSBA GMTs measured after the different MenC vaccination schedules (adapted from Pace et al. (2015))

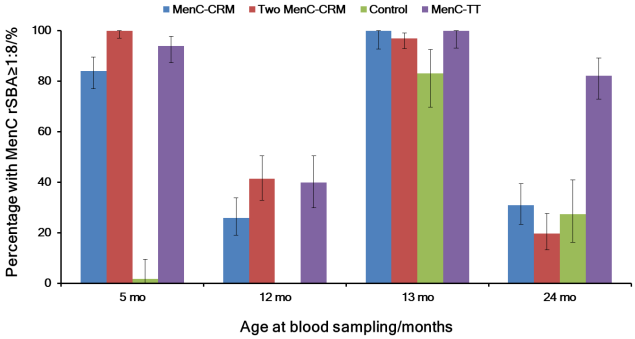

Figure 2: Percentage of participants with a MenC rSBA≥1:8 (error bars indicate 95%CI) (adapted from Pace et al. (2015))

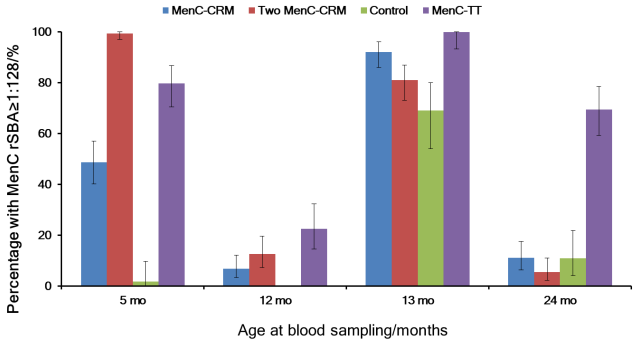

Figure 3: Percentage of participants with a MenC rSBA ≥1:128 (error bars indicate 95%CI) (adapted from Pace et al. (2015))

the mean log10 MenC rSBA between the single and two infant dose MenC-CRM197 Groups was 0.35 (95% CI 0.17 to 0.53) which not only showed that one dose was as good as two MenC-CRM197 doses but that actually a single dose was superior since the 95% CI of the difference did not cross 0 (Pace et al., 2015).

This study revealed differences in the immunogenicity between the MenC vaccine schedules and formulations studied. Two doses of the MenC-CRM197 vaccine in infancy resulted in higher seroprotective rates (taken as MenC rSBA titre of 1:8 or higher with a titre of 1:128 or higher being a more conservative estimate of protection) and GMTs compared to the single dose schedules at 5 months of age (100% vs 84.03%, p≤0.00001 for MenC rSBA≥1:8 and 99.27% vs 48.61%; p≤0.00001 for MenC rSBA ≥1:128 with GMTs: 620.54 vs 53.56; p≤0.00001 when compared to the single dose MenC-CRM197 group and 100% vs 93.94%; p=0.004 with MenC rSBA 4≥41:8; 99.27% vs 79.80%; p≤0.00001 with MenC rSBA≥1:128 and with MenC GMTs of 620.54 vs 169.37; p≤0.00001 when compared to the single dose MenC-TT group (Pace, 2015). By 12 months of age GMTs as well as the proportion of participants with seroprotective titres in all groups had decreased and only 25-41% had MenC rSBA≥1:8 (figure 2) (Pace et al., 2015). Those primed with a single MenC dose had significantly higher GMTs compared to those primed with 2 doses in infancy, one month after the 12 month booster dose (MenC GMTs 660.6 and 2779.2 in the single dose MenC-CRM197 (p=0.0001) and MenC-TT groups (p<0.00001) respectively vs 295.4 in the two dose MenC-CRM197 group) (figure 1) (Pace et al., 2015). One month after the Hib-MenC-TT vaccine boost the proportion of children with rSBA titres ≥1:8 was not significantly different between the Men C primed groups although again those with MenC rSBA titres ≥1:128 were significantly higher after a single MenC priming dose (figures 2 and 3) (Pace et al., 2015).

By 24 months of age the proportion of children with MenC rSBA ≥1:8 declined to <31% in the single or two dose MenC-CRM197 groups and in the control group (figure 2). In contrast the proportion of children with MenC rSBA≥1:8 and ≥1:128 were significantly higher at 82.1% and 69% respectively in those who had been primed with one dose of MenC-TT in infancy and boosted with the Hib-MenC-TT vaccine (p≤0.0001) when compared to the single or two dose MenC-CRM197 groups and the control groups (figure 2) (Pace et al., 2015).

In this study the iantibody dynamics following a conjugate MenC vaccine booster dose as an indicator of immune memory was also studied (Pace et al., 2016) in contrast to previous studies when classically a pure polysaccharide MenC vaccine was used to assess the response to a booster dose several months after the primary vaccination schedule. The practice of boosting with a pure polysaccharide vaccine has raised concerns that even with fractional doses the resultant antibody levels were lower than those induced with primary vaccination, a phenomenon called hyporesponsiveness: this could translate clinically

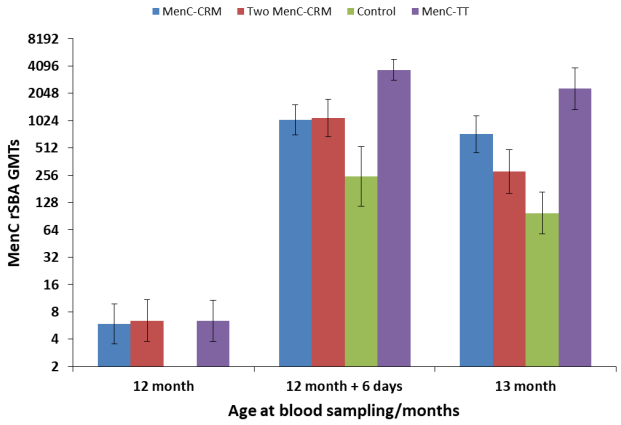

Figure 4: MenC rSBA GMTs at 12 months, 12 months+6 days and at 13 months according to the priming MenC infant schedule (adapted from Pace et al. (2016))

in a lower antibody response when subsequently exposed naturally to the invasive pathogen with possibly an increased risk of infection rather than protection (Gold et al., 1977; MacDonald et al., 1998). Paired sera from the 12 month pre-boost and 6 days post the Hib-MenC-TT vaccine boost were available from 180 participants (Pace et al., 2016). Priming with any MenC vaccine schedule in infancy resulted in significantly higher proportion of participants with MenC rSBA≥1:8 (100% vs 82.6%; p=0.001) and MenC rSBA GMTs (660.6; 295.3, 2779.2 in the single dose MenC-CRM197, two dose MenC-CRM197 and MenC-TT groups, respectively) compared to the control group (MenC rSBA GMTs 121.6; p≤0.00001) (figure 4 (Pace et al., 2016). The GMRs and after adjusting for pre-boost antibodies, the GMFRs, were similarly significantly higher in the primed compared to the unprimed groups (GMRs 4.2, 4.4 and 14.9 for the single dose MenC-CRM197 group, two dose MenC-CRM197 group and the MenC-TT group; GMFRs: 220.2, 229.9 and 778 in the single dose MenC-CRM197 group, two dose MenC-CRM197 group and the MenC-TT group, respectively compared to a GMFR of 57.7 in the control group) (Pace et al., 2016). The proportion of participants with MenC rSBA≥1:8 and ≥1:128 were similar between the MenC-TT and one/two MenC-CRM197 primed groups (100% in all groups with MenC rSBA≥1:8 and for MenC rSBA≥1:128: 100% vs 97.7% and 95.2% respectively), although the GMTs figure 4, GMRs and adjusted GMFRs were significantly higher in those primed with MenC-TT (Pace et al., 2016). No significant differences were seen with the single or two dose MenC-CRM197 primed groups.

An exploratory analysis of the antibody kinetics from the 6th to the 28th day post boost, performed in 162 participants showed a drop in MenC GMTs in all groups figure 4) with the adjusted GMFRs, now expressed as a decline, as well as the adjusted GMRs being significantly less in those unprimed in infancy compared to those who were primed with any of the schedules (Pace et al., 2016). Differences were again noted between those primed with MenC-CRM197 compared to those primed with MenC-TT with the adjusted GMRs being significantly higher following MenC-TT priming (0.48 compared to the single infant dose MenC-CRM197 group; p=0.039 and 0.2 when compared to the two infant dose MenC-CRM197 group; p≤0.0001) (Pace et al., 2016).

4.3 Safety

The most frequent local side effects following the 3 month vaccines were erythema in 45%, induration in 25%, pain in 22% and swelling in 16% reported in any of the groups receiving a MenC conjugate vaccine and with no significant difference seen between the different groups, excluding the control group (Pace et al., 2015). The most frequent systemic side effects were sleepiness and irritability observed in 50% and 66% of infants in each group respectively and again with no significant difference being seen between the groups. Fever ≥38oC was very uncommon and was observed in ≤ 1% of participants in each group (Pace et al., 2015).

Following Hib-MenC-TT vaccination the most frequent local reactions were erythema in 76%, induration in 31%, pain in 29% and swelling in 23% in each group (Pace et al., 2015). The most common systemic side effects were irritability in 61%, drowsiness in 39% and diminished appetite in 35% in each group (Pace et al., 2015). No significant differences were seen between the groups.

4.4 Relevance of the study findings

On an individual level, protection against the meningococcus is critically dependent on having an rSBA GMT ≥1:8 which has to be sustained (Auckland et al., 2006). The reason is that the 24 hour to 7 day incubation period of the meningococcus (De Wals et al., 1981) is shorter than the time needed for the immune system to respond following exposure, which is 9 days for a person who has no detectable MenC antibodies but who has become colonised with the meningococcus (Edwards et al., 1977) and 5-7 days in those who have been vaccine primed and subsequently challenged (Findlow et al., 2011; Snape et al., 2006). Having low MenC rSBA titres would make one potentially susceptible to IMD if exposed (Cano et al., 2004; Trotter et al., 2004). Generating high MenC rSBA GMTs following vaccination is important since it signifies a higher percentage of individuals with MenC rSBA titres ≥1:8. On a population level it is the percentage of individuals with MenC rSBA titres ≥1:8, and more conser-

vatively ≥1:128, that is important in the control of MenC disease (Auckland et al., 2006), the accepted proportion of which depends on the incidence of MenC disease in that same population.

It is a fact that vaccine schedules against meningococcal disease differ between countries with respect to the number of priming doses used in infancy, varying from none to two doses followed by booster doses in early childhood or adolescence (European Centre for Disease Prevention and Control, 2021). This shows that the scientific evidence behind planning of MenC vaccine schedules is not robust enough to result in a standard MenC vaccination programme in all countries where MenC disease is endemic. Immunogenicity data from this randomised controlled trial directly support the reduction of two infant priming MenC doses to one, so long as a booster dose is given around 12-13 months of age. Changes to a vaccination programme would also need to consider the dynamic epidemiology of the disease that is being prevented. Data from this trial support the reduction of infant MenC priming doses from two to one dose, with the retention of the 12 month Hib-MenC boost that was implemented in the UK since 2013 (Public Health England). This was a time when infant MenC disease was low because of the effectiveness of a previous catch up MenC vaccination campaign that led to disease control but with the simultaneous introduction of a MenC conjugate vaccine boost in adolescents, subsequently replaced in 2015 with a MenACWY conjugate vaccine due to a rise in MenW disease in adolescents, to prevent transmission to younger children (Public Health England and National Health Service England, 2015). This study also supports the complete removal of the MenC infant dose adopted in the UK since 2016 since one dose of the Hib-MenC-TT vaccine at 12 months without previous priming also results in robust immunogenicity at 82% with rSBA titres ≥1:8, although such a change was only performed when infant MenC disease was very low due to herd protection which was being sustained through adolescent vaccination. The effectiveness of a MenC vaccination programme adopted in the Netherlands, with a single dose at 14 months of age following control of MenC disease through a catch up campaign of older children and adolescents supported this decision (Kaaijk et al., 2012).

This study also revealed immunogenicity differences induced by different MenC conjugates or by schedules utilising repeated doses of the same MenC glycoconjugate vaccine. Giving two priming doses of MenC-CRM197 in infancy results in a significantly less post boost MenC GMTs after a 12 month Hib-MenC-TT vaccine compared to a single MenC-CRM197 prime and boost schedule. Although not reflected in the number of memory B cells circulating in the blood post MenC vaccination (Khatami et al., 2014), this could be a result of a difference in the amount of B cells induced in lymphoid tissue which may be less when a higher concentration of MenC-CRM197 (by giving more than one dose) is used for priming (Pace et al., 2015).

A single MenC-TT dose at 3 months resulted in significantly higher MenC rSBA GMTs compared to a single MenC-CRM197 dose. In addition the MenC antibodies measured after the 12 month Hib-MenC-TT boost were again significantly much higher in those primed with MenC-TT (Pace et al., 2015). Such differences could be due to the type of carrier protein used (a carrier protein is the protein to which the polysaccharide is attached to), specifically tetanus toxoid being a stronger immunogen than CRM197 (Richmond et al., 2001) when used for priming as well as when the same TT carrier protein is used for priming and boosting. The greater number of memory B-cells measured in subjects primed with MenC-TT and boosted with Hib-MenC-TT compared with those primed with MenC-CRM197 further strengthens this argument (Khatami et al., 2014). However, differences in the conjugation chemistry, the type of MenC oligo/polysaccharide used in the vaccine as well as other differences in vaccine formulations between different manufacturers could explain immunogenicity differences seen between the different MenC glycoconjugates. Such observations can determine the MenC vaccine formulation used for priming and boosting infants when a MenC vaccination schedule is planned.

This study was the first to demonstrate the use of a MenC glycoconjugate vaccine as a probe for immune memory (Pace et al., 2016), when classically previous studies used a pure polysaccharide MenC vaccine formulation to challenge primed subjects, a practice that raises concerns on the induction of hyporesponsiveness. MenC priming in infancy is important to generate high post boost MenC rSBA GMTs as shown by the higher proportion of subjects with MenC rSBA GMTs ≥1:8 and higher MenC rSBA GMTs in those primed in infancy compared to those who received their first MenC conjugate vaccine dose at 12 months of age (Pace et al., 2016). The magnitude of MenC rSBA GMTs, GMRs and adjusted GMFRs at 6 days post boost may be used to distinguish primed from unprimed children. Again differences in the immune kinetics were seen between the different MenC conjugate vaccine prime and boost schedules used with significantly higher MenC GMTs (figure 4), GMRs and adjusted GMFRs seen at 6 days after the Hib-MenC-TT boost in those primed with MenC-TT compared to priming with a single or two dose MenC-CRM197 vaccine schedule in infancy. Antibody decline from the 6th to the

28th day post the Hib-MenC-TT boost was also slower in those primed with MenC-TT compared to MenC-CRM priming (figure 4) (Pace et al., 2016).

5 Practical significance of the study data

How can the findings of this trial be implemented on a practical level? In an epidemiological study by Pace et al. (2020) looking at meningococcal disease in Malta over an 18 year period, from 2000- 2017, it was demonstrated that infants had the highest age specific incidence rates of IMD at 18.9/100,000 population with the incidence of capsular group B, C and W disease being significantly higher than in all other age groups. Furthermore, although there was a declining trend in MenB disease in the population, reflecting natural variation in MenB disease, the overall incidence of IMD remained stable. This was a result of the stable incidence of MenC disease as well as the appearance of MenW and Y disease in the population (Pace et al., 2020). The overall stability of the incidence of MenC disease (0.25/100,000 population from 2000-2008 compared to 0.33/100,000 population from 2009 – 2017) was in sharp contrast to the declining incidence of MenC disease in Europe dropping significantly from 0.22 to 0.1 over the same time periods as a result of the MenC vaccination programmes introduced within several European countries as discussed by Pace et al. (2020). This demonstrated the urgent need to introduce a MenC, as well as a MenB vaccination programme, in Malta. The single prime and boost MenC vaccine schedule introduced in Malta in 2020 is again supported by the findings from the study above, although the findings were extrapolated to the use of a MenACWY conjugate vaccine, which was more pragmatic considering the appearance of MenW disease in infants and MenY disease in adolescents (Pace et al., 2020). Although the impact of this schedule on MenC disease is still to be seen, a one-time catch up campaign would have been crucial to induce herd protection against MenC disease, as observed in other countries.

6 Conclusions

Vaccines are an extremely important tool in the prevention of infectious diseases. The way vaccines are scheduled on immunisation programmes is a dynamic process that reflects the availability of immunogenicity and safety data from vaccine trials as well as the epidemiology of the infectious diseases the vaccines are aiming to prevent, especially for those infections which are prevalent in children. This underlines the importance of conducting clinical vaccine trials in children. Having a vaccine research centre in Malta would facilitate collaboration in vaccine trials being conducted in Europe and would push Malta to the forefront of clinical vaccine research.

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Review Article

Research on Microgrids at the University of Malta

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Abstract. This article presents some of the work done in recent years by the microgrids research team at the Department of Industrial Electrical Power Conversion (IEPC). Research activities are dedicated towards enabling secure, reliable, and carbon free electricity systems. To date, the main contributions by the department were made to the operation, control and management of microgrids in low voltage (LV) distribution networks, ship electrification (shipboard microgrids) and low voltage DC microgrids. The paper also presents a few of the significant results achieved by the department to date including a hierarchical control architecture for single phase microgrids, control algorithms of power electronic converters for AC and DC microgrids, energy and power management strategies, power quality improvement algorithms and technologies, demand response strategies and devices, and the effective integration of renewables and energy storage systems.

Keywords: Microgrids, Electric Transportation, Demand Response, Power Quality

1 Introduction

The decentralisation of energy generation is a recent phenomenon caused by the wide-scale integration of distributed renewable energy sources (RES). RES in low-voltage grids (e.g. small-scale photovoltaic systems) generate energy closer to the consumers. The instantaneous penetration of RES can vary considerably even during the course of the day due to the end consumers usage patterns and due to the intermittent and variable output of the RES. For example, without factoring in the intermittency due to cloud coverage, the integration of photovoltaic (PV) generation affects dramatically the shape of the net-demand curve due to its variable output. PV generation increases the system's downward ramp in the morning and the upward ramp in the evening causing the so-called "camel curve" net-demand characteristic to change into the "duck curve". High instantaneous PV penetrations might also create an oversupply situation in the middle of the day, resulting in a negative net demand (i.e. reverse power flow). In addition, if the instantaneous penetration of RES were more than 50%, the system would be operating as an inverter-dominated grid with the corresponding challenges of stability, quality and reliability (Kroposki et al., 2017).

Microgrids are self-contained electricity distribution networks in which the RES together with energy storage systems (ESS) and local loads work cooperatively as a single local system and typically have a single point of common coupling (PCC) as an interface with the main grid. Microgrids can either operate autonomously in stand-alone (islanded) mode or they can be integrated into the present distribution networks (grid-connected operation) without the need to modify the existing power systems. Microgrids also offer a solution to remote electrification (i.e. provide electricity in areas where grid connection is not a viable option) and offer better supply reliability to the end user when compared to the conventional electric grid. Microgrids (AC, DC or hybrid AC/DC) are key elements for integrating high levels of renewable energy resources as well as distributed energy-storage systems as these maximise energy efficiency and improve the reliability of the local electrical network.

This article presents some of the research work performed in recent years by the microgrids research team at the Department of Industrial Electrical Power Conversion. The department has been applying microgrid concepts and technologies to a number of applications by cooperating with academia and industrial partners. The rest of the paper shall present a few of the significant results achieved by the department to date and is organised as follows, with each section focusing on a specific topic. Section 2 describes the hierarchical control structure for control and management of microgrids and mi-

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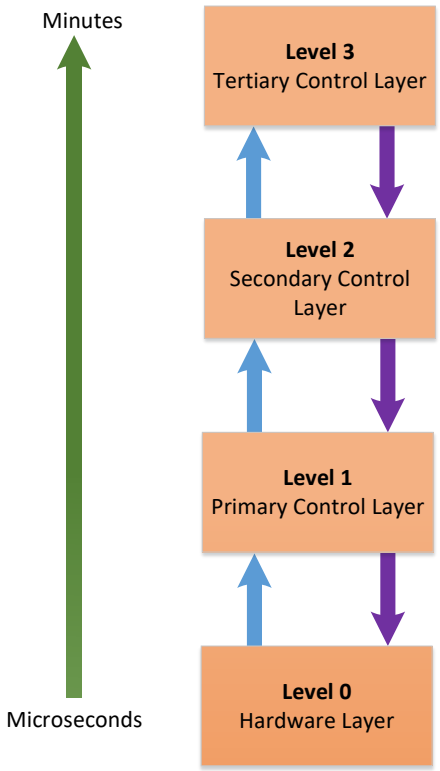

Figure 1: Hierarchical control architecture for microgrids.

crogrid clusters. Section 3 describes research activities on single phase microgrids while Section 4 describes the activities on low voltage DC microgrids. Section 5 deals with microgrids for the electrification of transportation while Section 6 describes the demand response strategies and devices for microgrid applications.

2 Control and management of microgrids

The hierarchical control strategy for microgrids is a widely accepted structure that consists of four distinct layers (Guerrero et al., 2011). The layers are shown in Fig. 1 where the bandwidth of the control loops is the highest at the physical level and decreases as the levels increase. Each layer has distinct roles for the effective implementation of grid-connected and stand-alone (islanded) microgrids.

2.1 Hardware layer

The power electronic converters (PECs) are the building blocks that enable the formation of the microgrid. The PECs convert power from one form to another and allow

Figure 2: A microgrid DC-to-DC converter implemented for the DC microgrid in the IEPC labs.

the formation of AC, DC and hybrid (AC/DC) microgrids. The PECs enable the connection of RES, ESS and various loads in the microgrids according to the selected network architecture. The converter level control (Level 0) within the PECs, determines the switching pattern for the solid state power semiconductors based on the operation of the inner voltage and current control loops. Three laboratory-scale setups have been developed by the department of IEPC to date for different network architectures. The first setup consists of a single phase AC microgrid consisting of three DC/AC inverters connected to a common AC grid that can work either in stand-alone or even in grid-connected operation. The second setup consists of a three phase microgrid consisting of two DC/AC inverters connected to a common AC grid that can work only in stand-alone mode. The final setup consists of a DC microgrid consisting of two unidirectional DC/DC converters and a bidirectional DC/DC converter connected to battery storage system. One of the DC/DC converters that was implemented for the DC microgrid in the IEPC labs is shown in Fig. 2.

2.2 Primary Control Layer

The primary layer (Level 1) is the application layer, implemented within the PEC, that uses local information to impart basic microgrid functionality and provides the reference set points for the PEC inner control loops. The role of the primary control loop is to maintain the balance between supply and demand to ensure that the microgrid remains stable under varying load conditions. A block diagram of the primary control loops of the DC/AC

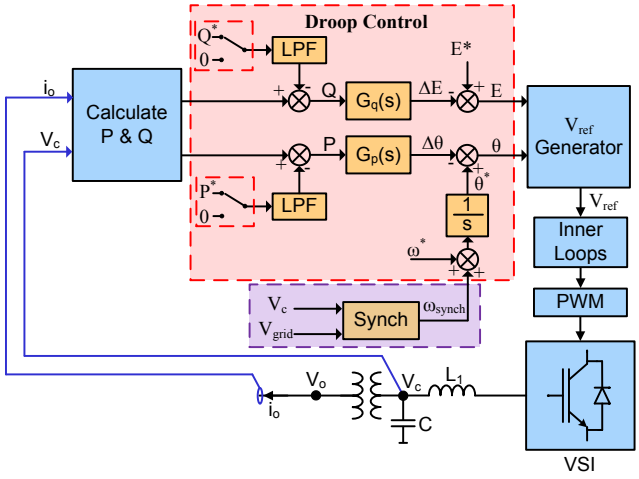

Figure 3: Block diagram of the primary control loops of the DC/AC power electronic converters for single phase microgrids.

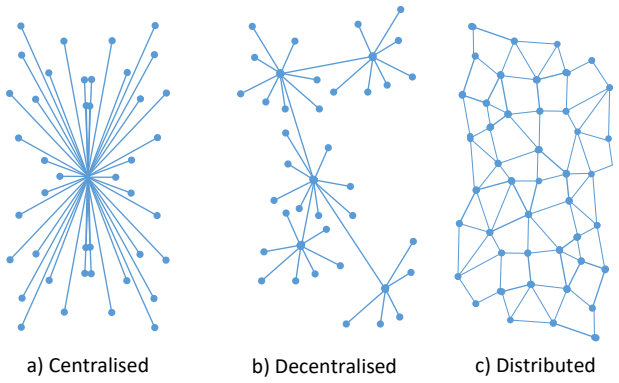

Figure 4: Representation of Centralised, Decentralised and Distributed microgrid architectures.

power electronic converters for single phase microgrids is shown in Fig. 3. The PECs that are deployed in either AC, DC or hybrid microgrids typically use droop control in the outer loop for distributed power sharing. The droop strategy varies according to the type of microgrid and it enables the parallel operation of PECs without any communication among the PECs albeit having a number of limitations (Micallef et al., 2014). This layer also includes virtual impedance loops in the inner loops of the converters that are typically used to improve the power sharing. Virtual impedance loops emulate various complex impedance behaviours and can be applied in both single-phase, three-phase and DC microgrid PECs, albeit with some important differences (Micallef et al., 2017a).

2.3 Secondary Control Layer

The secondary control layer (Level 2) implements the control and management algorithms for the optimal operation of the microgrid. The secondary layer provides the reference set points for the primary control loops. Algorithms in this layer are concerned with energy balancing of ESSs, black start management, power quality improvement, synchronisation voltage and frequency restoration. The secondary layer also coordinates the transition from grid-connected to islanded operation and vice-versa. The secondary control functionality can be implemented either as centralized, decentralized (or quasi centralised) or distributed, as shown in Fig. 4, with each architecture having its own advantages and limitations. In a centralized system (Fig. 4a), the RES, ESS and loads are all connected to a central microgrid controller (MGCC). The MGCC stores data and user information to assign set-points to each entity within the microgrid so as to attain the microgrids goals. In a centralised system, the main limitation is that the MGCC is a critical point of failure. Decentralized systems (Fig. 4b) do not have one MGCC but they use multiple central controllers, each of which usually assigns set-points to each entity within the microgrid. The decisions are taken locally as the different actors usually have different goals. A decentralized system can be just as vulnerable as the centralised but it is more tolerant to faults. Finally, the distributed system (Fig. 4c) is similar to the decentralized, but it eliminates centralization. In a distributed system, all entities have equal rights and decisions are taken through consensus among the all the actors.

2.4 Tertiary Layer

The tertiary control layer (Level 3) is the highest level of the hierarchy that implements energy management and power flow control strategies. The tertiary layer supervises the operation of the microgrid, regulates the power import/export from the microgrid, coordinates the operation of microgrid clusters, implements energy management strategies that optimise single or multiple variables (cost, efficiency, etc..). The tertiary layer is implemented as a centralised architecture since the decisions at this level impact the whole microgrid or microgrid clusters.

3 Single Phase Microgrids

Renewable energy sources are being integrated into single-phase low-voltage distribution networks to produce energy closer to the consumer. The formation of low-voltage microgrids can achieve high-energy efficiency and can also increase the reliability of the electrical supply. However, the combined power injected by the local RES into the grid can cause power quality issues during scenarios where the generation exceeds the demand. In Micallef et al. (2015)

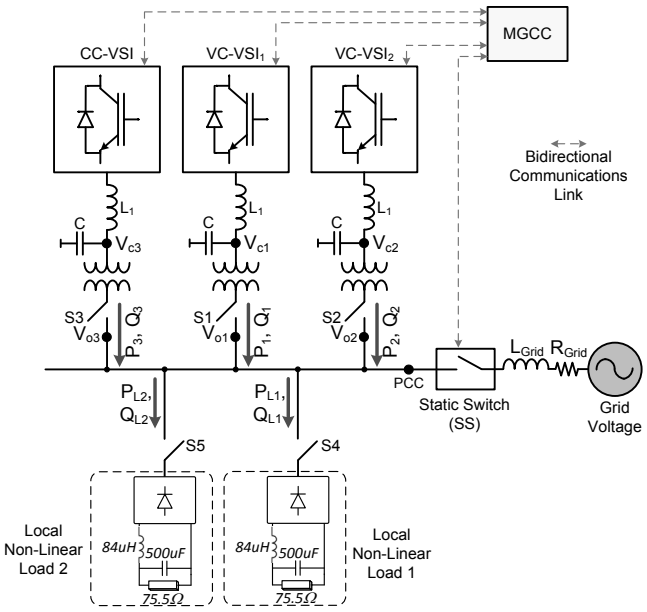

Figure 5: Block diagram of the AC single phase microgrid topology by IEPC (Micallef et al., 2015).

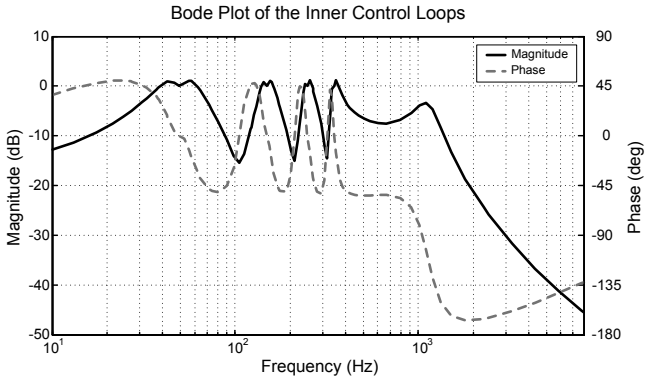

Figure 6: Bode plot of the inner control loops of a microgrid inverter with proportional resonant controllers (Micallef et al., 2017a).

and Micallef et al. (2017a), we designed, simulated and implemented a single phase microgrid that emulates a group of neighbouring households in a residential area that are connected together to form a microgrid. A block diagram of the considered microgrid topology is shown in Fig. 5. Research activities by the department have addressed the limitations of the decentralized operation of parallel PECs using conventional droop control including the voltage and frequency deviations, fundamental and harmonic current sharing. Solutions to overcome its inherent limitations and optimize the performance of the microgrid were also designed, modeled and experimentally verified. A summary of the main contributions shall now follow.

3.1 Power Quality Mitigation

The authors have highlighted the main power quality issues related to single-phase microgrids in Micallef (2019), together with a critical review of methods and algorithms to mitigate these phenomena. While various research studies have been proposed in literature concerning power quality mitigation in three-phase microgrids, not all of these solutions can be applied directly to their single-phase counterparts. Power quality issues of consequence that were identified for single phase microgrids include: reactive power exchange; voltage and frequency fluctuation; and current and voltage harmonic distortion.

In Micallef et al. (2017a), we proposed selective harmonic control loops by proportional resonant controllers that are implemented in the primary control layer of the PECs themselves in single-phase microgrids.The proportional resonant controllers adapt to the varying droop frequency since the frequency of the microgrid voltage varies due to the droop control. An additional advantage of proportional resonant controllers is that selective harmonic control can be integrated into the controller quite easily by cascading additional resonant transfer functions tuned at the required frequencies. An example of the frequency response of the inverter inner control loops with proportional resonant controllers is shown in Fig. 6 where bandpass characteristics are introduced in the frequency response at the 3^{rd} (150Hz),5^{th} (250Hz) and 7^{th} (350Hz) harmonic in addition to the fundamental frequency (50Hz).

Virtual impedance loops in literature were based on resistive and/or inductive impedances that degrade the voltage harmonic distortion at the PCC to improve the fundamental power sharing. Thus, we proposed a resistive-capacitive virtual impedance loop in Micallef et al. (2014) and Micallef et al. (2017a) that improves the power sharing among the PECs, reduces the harmonic currents output by the RESs and also improves the voltage harmonic distortion at the PCC. The resistive-capacitive virtual impedance loop in principle emulates a virtual capacitive bank connected in series with the output of the inverter as shown in Fig. 7. The capacitive virtual impedance reduced the voltage total harmonic distortion at the PCC by 22.7% during experimental tests with an improvement in both the fundamental and harmonic current sharing.

In Micallef et al. (2015), we proposed secondary control loops shown in Fig. 8, that restore the microgrid voltage and frequency while eliminating the reactive power exchange between the PECs in single-phase microgrids. In addition, the primary and secondary control loops in Micallef et al. (2015) enable the microgrid to trans-

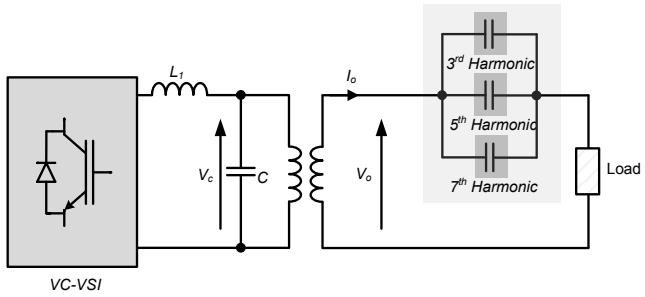

Figure 7: Schematic representation of the resistive-capacitive virtual impedance loop (Micallef et al., 2017a).

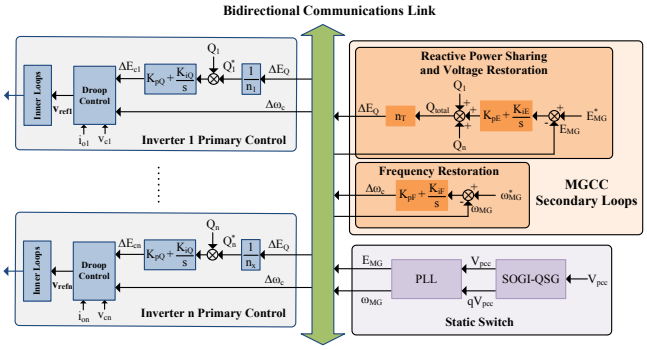

Figure 8: Secondary control loop for voltage and frequency restoration, reactive power sharing, and seamless transitions between grid connected and islanded operation. (Micallef et al., 2015).

ition from islanded to grid-connected operation and vice versa without any disconnection times for the consumer. The communication bandwidth is selected as a compromise between the total data that needs to be transferred through the network infrastructure and the transient response that is required from the secondary control loops. A high data bandwidth implies a fast transient response for the secondary control loops, while providing a high data transmission rates. Results have shown that low bandwidth communications can be used to achieve the required functionality with bandwidths as low as 1Hz. In Micallef et al. (2017b), we have also demonstrated how integrating RES in LV single phase microgrids can result in improved power quality of the electrical grid. A similar hierarchical architecture was employed for grid connected operation that avoids the voltage rise effect at the PCC from occurring by regulating the active and reactive power outputs from the PV inverters.

4 DC Microgrids

DC microgrids are gaining popularity due to their advantages over their AC counterparts. DC microgrids have lower conversion losses since PVs, batteries and

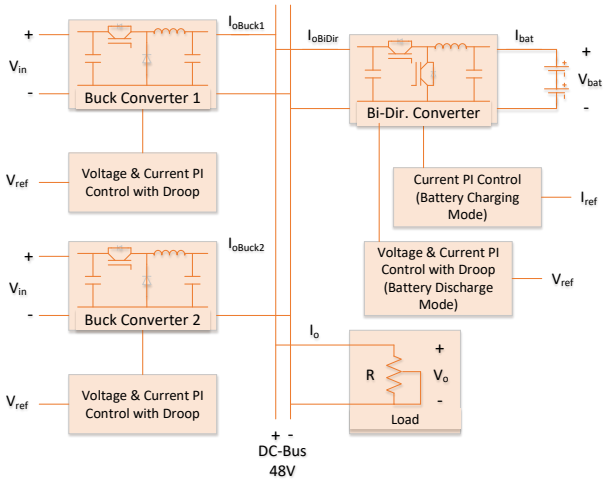

Figure 9: Block diagram of the DC microgrid topology by IEPC.

other RES have a DC output and thus these need less conversion stages (fewer PECs required). In addition, DC microgrids do not require any synchronisation or phase/frequency regulation. Another advantage is that the DC microgrid is not affected by power quality effects on the AC side (e.g. voltage sags, dips, etc..). These advantages make the DC microgrid attractive for numerous applications such as residential (behind-the-meter) installations, electric transportation (e.g. maritime microgrids), and offshore applications, amongst others.

For typical DC microgrid applications, the DC bus voltage is maintained by the utility through an AC/DC converter, while local loads and RESs are connected to the DC bus through DC/DC converters. The voltage level of the DC bus is dependent on the application and is regulated by the droop control loops implemented in the PECs. In Zammit et al. (2016), we describe the design of the primary control loops for parallel step down (buck) DC/DC converters in a behind-the-meter DC microgrid with conventional droop. For this application, a 48V DC bus was selected since this voltage level is considered inherently safe ("Ieee standard for dc microgrids for rural and remote electricity access applications", 2021) and does not require any additional safety considerations for behind the meter applications. However, should the residential building require high power consumption, then the bus voltage should be increased to reduce the system losses. In addition, we have also integrated a secondary voltage restoration loop to keep the desired voltage in the DC microgrid for varying load conditions. In Zammit et al. (2019) and Zammit et al. (2020), we proposed a novel droop control method termed as combined voltage and droop (CVD) for DC microgrid applications that is

built on the I-V droop method. The three droop methods were designed, modelled and simulated to compare their operation and performance where the proposed loop obtained faster dynamic operation without compromising the power sharing capabilities. The converters were then designed, modelled and built to form an experimental DC microgrid setup. A block diagram of the DC microgrid topology by IEPC is shown in Fig. 9.

5 Transportation Electrification

The international drive towards maximizing fuel efficiency is accelerating the development of battery-hybrid vessels, while the integration of alternative fuels (e.g hydrogen, ammonia, etc..) is still in the early stages. Hundreds of ships with installed batteries are already in operation, including numerous retrofits and upgrades. Marine batteries are still undergoing rapid development and can provide various benefits even though they cannot presently solve the challenges related to long distance shipping. Short distance ferries are one of the few segments that have already seen uptake in both all-battery powered and hybrid solutions. Alternative fuels (e.g. hydrogen, methane) are showing great promise for long distance shipping although significant effort is required to scale up these technologies.

5.1 Maritime Microgrids

Maritime vessels (ships, ferries, tugs, etc..) tend to be one-offs or built as a small series, tailored for their specific owner, operator and purpose. The challenge is to choose the right power system topology and equipment for each vessel according to the specific operational profile thereby minimising emissions and maximising energy efficiency. Modern vessels adopt integrated power systems architectures, connecting the generators, propulsion and other loads to a common AC or DC bus (Sulligoi et al., 2016). The electrification of ships due to the integration of batteries and alternative energy technologies (e.g. hydrogen fuel cells), is transforming ship power systems into highly dynamic microgrids. Microgrids onboard maritime vessels (also defined by maritime microgrids in literature) operate in islanded mode when the vessels are out at sea, and in grid-connected mode when these are connected to the shore-side electricity. An example of a maritime microgrid with a DC bus architecture is shown in Figure 10.

We have applied control strategies and technologies used in terrestrial microgrids to the marine sector, such as voltage and frequency control algorithms, power quality improvement strategies, power-sharing methods, and energy management strategies. Our recent work has focused on the modelling and simulation of the electrical

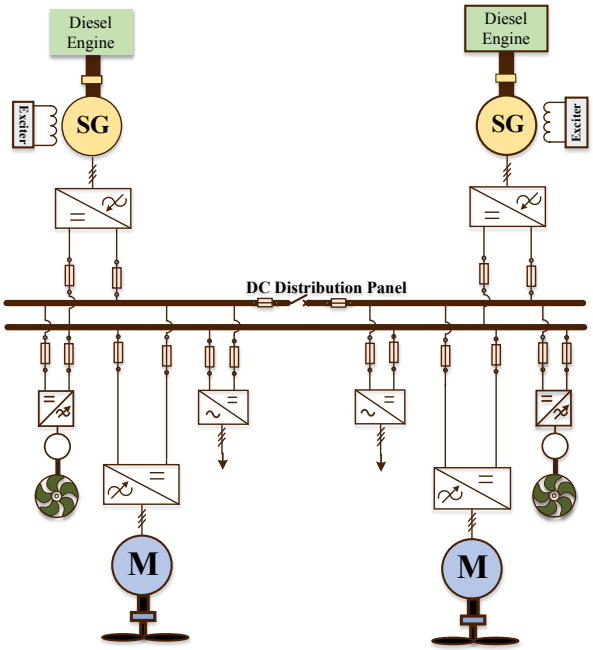

Figure 10: A line diagram of a modern maritime microgrid (Micallef et al., 2021).

power system of a hybrid MVDC ferry (Micallef et al., 2021). A DC bus architecture provides a number of advantages such as: (1) the diesel gensets can achieve higher efficiency (reduction in fuel consumption) since the AC/DC converter enables the gensets to run at variable speed; (2) simpler integration of battery energy storage since only DC/DC conversion is required; (3) faster and simpler parallel connection of gensets; and (4) reduced transmission losses. Rectifiers have fundamental importance in these architectures since this equipment provides the conversion from the AC voltages of the generator to the DC bus voltage. Therefore, these converters have a direct impact on the quality of the DC bus voltage. In Micallef et al. (2021), we have considered in detail the operation of an active front end (AFE) with LCL filter and a 12-pulse series-type diode rectifier and compared their performance with respect to DC voltage regulation capabilities and AC-side current harmonics. The hybrid MVDC ferry microgrid shown in Fig. 10 (excluding the bow thrusters) was modelled and simulated, whereby the parameters for the model were extracted from the datasheets of real equipment as provided by the industrial partner. The AFE was seen to exhibit better voltage regulation capabilities due to its fast control loops proving more effective than the 12-pulse rectifier during load changes. By feeding the DC link voltage directly into the automatic

voltage regulator (AVR), the generator excitation for the 12-pulse series rectifier implementation is adjusted to keep the voltage at the desired reference. During this time, the DC bus voltage shows an underdamped response with maximum voltage variations of up to 100V peak-to-peak, that could potentially destabilize the shipboard power system. On the other hand, the AFE shows faster voltage regulation capabilities with negligible DC bus voltage variations due to the designed fast control loops. The current output at the fundamental frequency (60Hz) is at 973.5A and 1007.6A for the 12-pulse and AFE respectively. The AFE also does not exhibit low-frequency harmonics and only shows switching frequency harmonics centred around the switching frequency, at 4.88kHz (2.92%) and 5.12kHz (2.79%), respectively. The resulting inverter-side current THD is of 4.4% while the generator-side THD is at 0.9%. The 12-pulse series rectifier has low frequency content at harmonic numbers 12K±1, however the transformer arrangement removes the 5^{th} and 7^{th} harmonics typically present in 6-pulse rectifiers. The 12-pulse rectifier spectrum resulted in harmonic frequencies at the 11^{th} (7.53%), 13^{th} (5.1%), 23^{rd} (1.34%) and 25^{th} (1.15%). The resulting generator-side current THD is of 9.3%. Both rectifiers show a significantly improved performance when compared with the conventional 6-pulse rectifier, however these advancements come at a higher complexity and cost. With the introduction of energy storage, hydrogen fuel cells and renewables on maritime vessels, maritime microgrids are transitioning towards a hybrid AC/DC power distribution architecture. In this context, we are presently collaborating with industry and academia towards the effective integration of batteries and alternative fuel systems in maritime microgrids.

6 Residential Demand Response

Demand response (DR) aims to manage the electricity demand such that this matches the available energy resources without adding new generation capacity (Haider et al., 2016). In Chen et al. (2017), the DR strategies are categorised as either direct (incentive based) or indirect (price based) as shown in Fig. 11. Through direct demand response programs, the consumers are offered incentives such that the distribution system operator (DSO) has remote control of participants' appliances (e.g. heating, ventilating, and air conditioning (HVAC), water heaters, or pool pumps) during certain hours of the day. Indirect demand response programs modify the consumer behaviour through price-based demand response strategies (e.g. time-varying price signals) to reduce energy usage during peak hours. Hence, end-users have a central role since these have to modify their habits by shifting their electricity consumption.

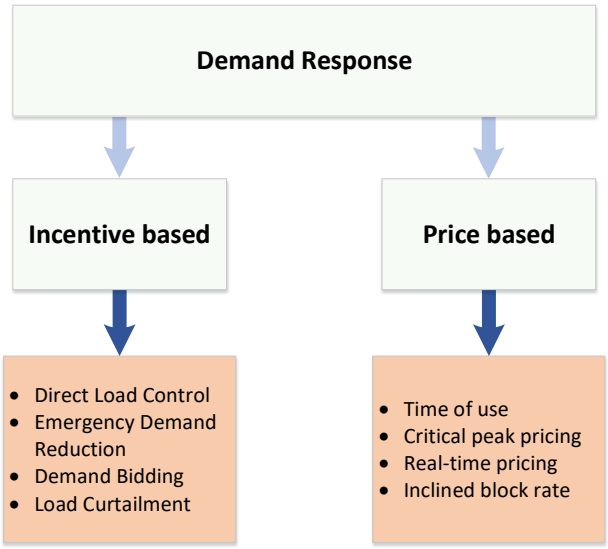

Figure 11: A block diagram summarising the demand response strategies.

The electricity consumption data and the corresponding load profiles are vital for the implementation of demand response programs. In Settino et al. (2019), we presented a detailed analysis of the electrical consumption and PV generation profiles of a Maltese dwelling. The monitoring was carried out for over one year at a resolution of 30 seconds for both the electricity consumption and the electricity generated by a photovoltaic system installed on the roof-top of the dwelling. The electrical demand varies widely on a day-to-day basis and has also seasonal variability thus making any accurate predictions of the consumption very difficult. The PV generation profile is also dependent on the season and can be intermittent due to variations in cloud coverage. An example of the determined seasonal daily profiles is shown in Fig. 12. The figure shows the mean and standard deviation at 30 minute intervals for both the PV generation and consumption. The self consumption ratio over the evaluated period was of 53.5%, (i.e. 53.5% of the electricity produced by the PV system is directly used while the remaining 46.5% is injected into the grid.) Therefore, battery ESS play an important role in demand response programs to increase self-consumption and provide additional services to the local electricity network.

Storage systems when combined with solar generation technologies enable the implementation of demand side strategies without being limited by the available controllable devices (e.g heating, ventilation and air conditioning). Energy management algorithms to maximize self consumption can ensure that the local generation matches

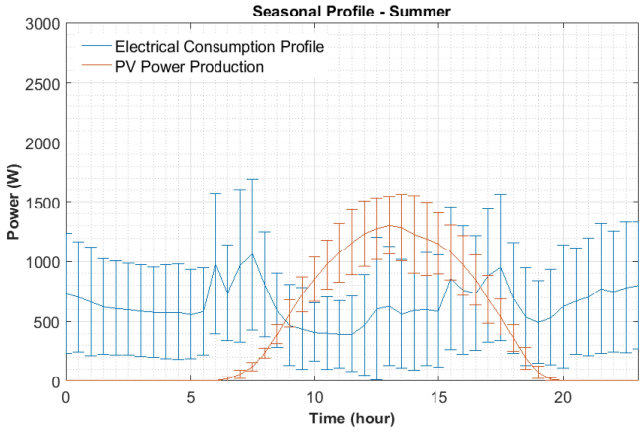

Figure 12: Summer seasonal daily consumption and PV generation profiles of a Maltese dwelling.(Settino et al., 2019)

the demand consequently reducing power variations and risks of over-/under-voltages. In (Settino et al., 2018), we performed a study of available solar technologies and storage systems. A comparison of photovoltaic systems coupled with solar thermal collectors, heat pumps and solar cooling technologies was carried out in terms of conversion efficiency, environmental impact and installation cost. The study determined that there is no definitive answer to which is the best overall technology. The performance of existing technologies strongly depend on the location, climatic conditions, loads (such as electrical and thermal) and existing policies. An in-depth analysis on a case by case basis should be performed to determine the best solution which suites the user's needs. A note worth mentioning is that PV systems with batteries show to have the lowest global warming potential (GWP), but the high initial cost of battery storage limits their usage. However, the prices for residential-scale battery ESS have reduced significantly in the past 5 years.

In Micallef et al. (2020), we proposed an electric spring-based (ES) residential smart water heater that can store excess PV generation as thermal energy. The block diagram of the considered LV microgrid and main household elements is shown in Fig. 13. The ES smart water heater is suitable for integration in both direct and indirect demand response programs. The ES smart water heater is a power-to-heat energy storage device that maintains the microgrid PCC voltage at the desired magnitude by controlling the active power absorbed by the water heater. The designed ES smart water heater is suitable for both grid-connected and islanded microgrid operation since the ES is synchronized with the PCC voltage. Simulations based on real PV and residential consumption data were used to verify the effectiveness of the proposed solution.

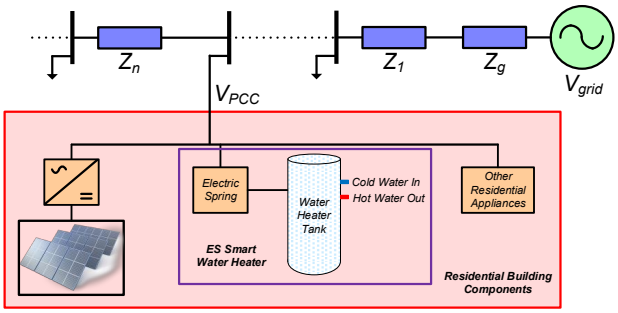

Figure 13: Block diagram of the considered microgrid scenario and main household components. V_{PCC} is the local PCC voltage being targeted by the operation of the ES smart water heater.

It can be observed that the ES water heater reduced the voltage at the PCC to the nominal voltage reference avoiding reverse power flow into the grid.

7 Conclusion

In the past decade, microgrids have gained a lot of traction as these play a key role to address modern-day energy challenges. There are still various open technical and non-technical (policy, regulation, economic) challenges and opportunities for widescale deployment. This article has summarised some of the research activities by the microgrids research team at the Department of IEPC. The presented work summarizes our contributions to the operation, control and management of microgrids in a number of applications.

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Research Article

Quality education, social cohesion and active labour market policies: A comparative analysis of two European island states

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Abstract. Societies benefit from the delivery of inclusive education, lifelong learning and from active labour market policies. Therefore, this research presents a critical review of the relevant literature. It features a comparative analysis on the latest socio-economic policies that are currently being implemented in the Mediterranean island states of Malta and Cyprus. The findings suggest that both countries need to attract more students to vocational and higher education in order to improve their employment prospects. The latest European reports indicate that their labour market policies are increasingly targeting vulnerable individuals, including women, single parents, older adults and migrant workers, among others, who are not in employment, education or training. In conclusion, this contribution implies that the pursuit of continuous improvements in quality education and social cohesion can create a virtuous cycle of productivity outcomes, including job creation and societal well-being.

Keywords: quality education, social inclusion, social cohesion, labour market, Malta, Cyprus, European Union, Coronavirus, COVID-19

1 Introduction

Education can contribute to create a fair and equitable society for all (OECD, 2008). It provides opportunities for social mobility as individuals are rewarded according to their own merit (Breen et al., 2005; Mok, 2016). Hence, educational and employment policies may play a significant role in shaping key performance indicators, to achieve social and economic outcomes (Dvouletý et al., 2016; Ramsden, 2003). Various, intergovernmental organisations, including the European Union, (EU), the Organization for Economic Cooperation and Development (OECD) and the United Nations (UN), among others, have recognised the importance of delivering excellence in education for the advancement of societies and economies. Arguably, the provision of quality education, may result in positive implications for job creation, competitiveness and prosperity (Camilleri et al., 2016; EU, 2014; OECD, 2012).

The United Nations Educational Scientific and Cultural Organisation (UNESCO), among others, suggested that quality education can improve the wellbeing of individuals and their families, whilst fostering better societies. UNESCO's 2003 policy document has reiterated the Delors Commission's 1996 recommendations for an integrated vision of education that provides learning opportunities for each individual to develop his or her full potential.

UN has dedicated a Sustainable Development Goal (i.e. SDG4) to raise awareness on the delivery of quality and inclusive education (i.e. SDG4) (Camilleri et al., 2020; UNSDG4, 2015; Vladimirova et al., 2016). Moreover, relevant theoretical underpinnings reported that higher standards of education would result in cohesive societies as well as economic growth and competitiveness (Gradstein et al., 2002; Green et al., 2003; Gupta et al., 2016; Thorbecke et al., 2002).

This contribution begins by exploring academic and non-academic literature, including regulatory guidelines and policies. It features a comparative analysis on education, social and labour market policies in the context of the island states of Cyprus and Mata. In a nutshell, the findings of this research suggest that both countries are responding to the EU's recommendations:

i. to reduce the number of early school leavers,
ii. to minimise the number of young adults and adolescents who are neither in education nor in employ-

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ment, and
iii. to entice individuals, including the most vulnerable ones, like single parents, unemployed adults and migrants, among others, to pursue higher, vocational education and lifelong learning opportunities, among other targets.

This contribution's underlying research question is: *To what extent and in which ways are education, social welfare and employment policies improving the social fabric as well as the economic performance in the Southern-European states of Cyprus and Malta?*
To the best of the authors' knowledge, there is no other academic contribution that clarifies how social cohesion and active labour market policies could affect the economic growth and competitiveness of small island nations (that are located in the periphery of Europe). Therefore, this research addresses this gap in academic knowledge and puts forward key implications to policy makers.

2 Literature Review

2.1 The provision of quality education for cohesive societies

Public education has been one of the main contributors to social cohesion in many countries (Green et al., 2003; Heyneman, 2000; Mickelson et al., 2012). Uniform schooling reduces re-distributional conflict among distinct groups in society and plays the dual role of building human capital and determining social orientation (Gradstein et al., 2000). Hence, several governments are investing resources, competences and capabilities in education to improve the quality of life of their citizens, including those hailing from the most vulnerable groups in society (Deacon, 2018).
The fourth United Nations' (UN) Sustainable Development Goal (SDG4) and its 10 targets represent an ambitious and universal agenda, that are meant to develop the individuals' skills for better lives. Five of these targets are concerned with improving the quality of education for individual children, young people and adults, to provide them with more competences for the labour market. During the last few decades major progress has been made towards increasing access to education at all levels, to instil individuals with relevant knowledge and skills for decent work and global citizenship (UNSDG4, 2015). SDG4 aims to eliminate gender disparities. It urges governments to provide equal opportunities for their citizens to access education and lifelong learning (UNSDG4, 2015).
A relevant review of the literature links most SDGs with education (Vladimirova et al., 2016) and social cohesion (Gupta et al., 2016). Notwithstanding, the promotion of quality education is already an important policy objective across many countries (Camilleri, 2017; Camilleri et al., 2016). For instance, Europe's 2020 Strategy was intended to improve the EU's competitiveness and productivity levels that underpin its economy (EU, 2010a, 2010b, 2020c). This strategy identified three priorities as the main pillars:

i. Smart growth (to develop an economy based on knowledge and innovation;
ii. Sustainable growth (to promote a more resource efficient, greener and more competitive economy); and
iii. Inclusive growth (to foster a high-employment economy by delivering economic, social and territorial cohesion)

(Pasimeni et al., 2015).
Europa 2020 aimed to increase the employment rates and to raise the quality of jobs, especially for the disadvantaged groups in society, including women, young adults and adolescents, disabled individuals and older workers (Gravani et al., 2019). It is also its intention to integrate migrants in the labour force. The latest European Policy Cooperation (ET2020) framework is based on a lifelong learning and social mobility approach. It addresses learning outcomes from early childhood to vocational and higher education for adolescents as well as for older adults. EU (2020c) specifies that its objectives are:

1. to improve the quality and efficiency of education and training,
2. to promote equity, social cohesion, and active citizenship, and
3. to enhance creativity and innovation, including entrepreneurial skills.

The EU Commission set reasonable targets to its member states to reduce their rate of early school leavers, and to increase the number of individuals who complete courses in tertiary education (EU, 2022a, 2022b). These targets are also consonant with the United Nations very own SDGs (Camilleri et al., 2020; UNSDG4, 2015). They have the potential to become a powerful political vision that can lead to a shared and long-lasting prosperity in different European contexts (Hajer et al., 2015).
In fact, the EU Commission articulated an action plan to integrate newly arrived migrants from third countries into mainstream education (EU, 2020c). "A cohesive society works towards the well-being of all its members, fights exclusion and marginalisation, creates a sense of belonging, promotes trust, and offers its members the opportunity for upward mobility" (OECD, 2011, p. 17). Education may well reduce any inequalities in society by fostering cognitive, interpersonal and emotional skills as well as promoting healthy lifestyles, participatory practices and

norms (Ayalon et al., 2004; Jackson, 2009). Therefore, the individuals' education as well as their ongoing training and development can improve their position in the social strata as well as their quality of life (Breen et al., 2005; Kilpatrick et al., 2003; OECD, 2012). Moreover, their countries' economic growth is closely linked with their capacity to create, retain and attract human capital (Forrest et al., 2001; Halpern, 2013) Hence, education policymakers need to anticipate and manage change by investing in skills and training programmes, whilst modernising labour markets and welfare systems.

In the past years, OECD's Programme for International Student Assessment (PISA[1]), as well as its adult version, the Programme for the International Assessment of Adult Competencies (PIAAC[2]) reported that although many countries are experiencing high attendances at schools and other education institutions; only a proportion of their students would eventually achieve adequate and sufficient levels of proficiency levels, when they complete their courses (OECD, 2018, 2019). Hence, bolder efforts are required to make even greater strides to deliver quality education for all (Camilleri, 2021).

These findings are exerting more pressure on education providers to meet their national performance criteria. Education institutions are expected to raise their students' learning outcomes through regular assessments, to improve the quality of their curricula and instruction, and to deal with children from diverse cultural and linguistic backgrounds (Ramsden, 2003; Timar et al., 2012). Some academic commentators argue that quality education ought to be affordable for all segments of the population, as it brings better prospects for upward social mobility and more inclusion in society (Goldthorpe et al., 2007).

2.1.1 Social Inclusion

Social inclusiveness has its roots in human rights, inequality, redistribution, entitlements and capabilities (Gupta et al., 2016). It involves empowering the most vulnerable individuals in society through investments in human capital, to enhance their participation in the labour market (EU, 2013c; Forrest et al., 2001). Social inclusion is non-discriminatory and is age-, gender-, caste-, sect- and creed- sensitive, in terms of income, assets and employment opportunities (Humphries, 2004; Liasidou, 2014). Education has the potential to bring social inclusion through civic and societal engagement (Putnam, 1995, 2001).

The schooling experience itself transmits common values that underpin social capital and social cohesion (Baldacchino, 2005; OECD, 2012). Green et al. (2003) argued that quality education acts in differential ways on both concepts. Their "distributional model" shed light on the relationship between the provision of fair education for all and the various measures of social cohesion. Green (2011) noted that Southern European schools in Spain, Portugal, Italy and Greece were not offering the same standards of education across their territories, as opposed to Nordic countries. He went on to suggest that the differences between schools was not driven by differences in social intake, but by the students' backgrounds. This had an effect on the students' performance.

Other authors, including Galston (2001) indicated that school-based efforts to form active citizens may not always be successful if the children's families and their local communities do not provide good opportunities for them to engage in civic activities. Similarly, Putnam (2001) argued that open classroom environments, classes that require practical involvement in social matters as well as the schools' ethos that promote active citizenship, can be conducive to building stronger civic participation, from a tender age. These efforts are most likely to be successful when community environments are aligned together with the institutional efforts made by policy makers (Estol et al., 2018).

The children's well-being and their social progress are more likely to work when their home and community environments are synchronised (OECD, 2010). In addition, quality education creates an inclusive schooling environment that can nurture social cohesive values towards the entire community (Flecha, 2014). Thus, students learn to become more inclusive toward other groups in society. The formulation of specific policies and measures for social equity can foster equal access to education for all. Efforts to close the gender gap in education may help to break the intergenerational transmission of poverty (Jacob, 2002). Therefore, policy makers are instrumental in emphasising the delivery of inclusive curricula and teaching practices that are aimed at fostering diversity in schools as well as in society (Ambe, 2006). An increased awareness among children on cultural and diversity issues would improve the integration of minorities in education, and eventually in the labour market. Inclusive schooling systems tend to perform better in terms of learning outcomes when compared to more segmented ones (Ainscow, 1997).

In reality, a significant fraction of children, mostly from disadvantaged households, are usually deprived access to quality education because they do not afford it (Currie, 2001; Liasidou, 2014). Alternatively, there may be other reasons why they may have missed the opportunity to develop their basic competencies, earlier on, in their life.

[1]PISA is OECD's widely used global metric to measure the quality of learning outcomes.

[2]PIACC is OECD's programme of assessment and analysis of adult skills.

Hence, the governments and their policy makers should adopt a more pragmatic stance to social equity issues in order to maximise the representation, participation and recognition of the disadvantaged groups in society, including older adults and migrants, among others (Humphries, 2004; Raffo et al., 2008).

2.1.2 Social Equality

Gradstein et al. (2002) maintained that education is a socialising force as it instils civic virtues from an early age. The provision of quality education facilitates the interaction between different demographic groups in society. As such, education has often played a key role in forging national identities and in establishing centralised governments. On the other hand, coercive, centralised schooling may result in less welfare than decentralised education (Deacon, 2002; Gradstein et al., 2002). The delivery of quality education and its relationship with economic growth is also conditioned by cultural and religious divisions (Gradstein et al., 2002; Spring, 2017). The distribution of ethnic groups and the social distance between them can affect this relationship (Gradstein et al., 2002). Hence, the design and assessment of educational reforms should take into account their impact on the socialising role of education.

Green et al. (2003) posited that education affects the socialisation of individuals as the schools' ethos and their curricula are conducive to social cohesion. The provision of an inclusive, quality education can lead to improvements to the individuals' communication and transferable skills, as it facilitates their cross-cultural understanding and civic participation. Thorbecke et al. (2002)'s study had indicated a strong correlation between the skills' distribution and income inequality across countries. They found a highly negative and significant relationship between educational inequality / income inequality with social cohesion. In a similar vein, Green et al. (2003) reported that educational inequality exercised a significant, negative effect on social cohesion; whilst quality education was related to social cohesion. The latter generates equal opportunities, in terms of income and cultural capital amongst different people.

Previously, Knack et al.'s 1997 study reported that trust and civic norms are stronger in advanced economies. In a similar vein, Green et al.'s 2003 empirical studies had proved that social cohesion and quality education are highly sensitive to inequality. Perhaps more attention ought to be placed on the development of shared or co-operative values and on the attenuation of inequalities in order to improve educational outcomes. Green et al. (2003) hinted that many Anglophone countries were placing more stress on raising mean levels of achievement rather than on reducing inequalities.

Arguably, the provision of quality education could lead to significant benefits to the labour market and to the achievement of desired economic outcomes. However, when it comes to promoting social cohesion, there is clearly a case for prioritising the social inclusion of the most vulnerable people in society (EU, 2013c). For instance, Beauchamp-Pryor (2012) maintained that individuals with special needs ought to be involved in policy development. She suggested that barriers such as power sharing, as well as the traditional ideologies are increasingly being challenged by these individuals who want to become more active in the labour market.

Ultimately, the regulatory institutions' responsibility is to tackle inequality that polarises their societies (EU, 2013a). Greater income inequality stifles upward social mobility, thereby making it harder for talented and hard-working people to get the rewards they deserve (Goldthorpe et al., 2007). Generally, the societal and economic development of a country would usually reflect the different dynamics of its institutional policies. In this light, the following sections critically analyse, the educational, social welfare and employment policies of two Southern European states, namely, Cyprus and Malta:

2.2 Active labour market policies of Cyprus and Malta

Cyprus and Malta are two Mediterranean islands. They obtained their independence from the United Kingdom in the 1960s. In 2004 they joined the European Union as fully-fledged member states. Both republics are service-based market economies.

2.2.1 Cyprus

EU (2020a) anticipated that the Cypriot economy was expected to continue growing, albeit at a slower pace, by around 2.8% in 2020 and by 2.5% in 2021, prior to the unprecedented outbreak of the Coronavirus (COVID-19) pandemic and before the Russian invasion of Ukraine. Cyprus' current account deficit was set at 10% of its GDP in the previous budget. Its unemployment stood at 7.5% in 2019, the lowest level since 2011, and this figure was expected to drop even further. Back then, its inflation was one of the lowest in Europe. Cyprus' economic structure and fiscal sustainability enabled it to invest in its public services, including on its national health insurance system, energy efficiency and renewable energy; research and development, et cetera. However, EU (2022a) noted that Cyprus has made limited progress in reforming its educational systems.

2.2.2 Cypriot education policies

Cyprus is striving in its endeavours to continue delivering quality education to its citizens, across all levels ((EU, 2020a, 2022a). In 2017, the Cypriot government has introduced a new recruitment system for teacher appointments that was based on competitive exams. As a result, in 2018 and 2019, 866 candidates were hired through its new system (EU, 2020a). Another deliverable that was incorporated in the strategic plan (2019-2021) of the Ministry of Education and Culture (MoEC) was focused on upgrading the learning content (EU, 2018c). This plan was implemented through the modernisation of curricula and timetable programmes, by improving the pupils' learning outcomes, introducing up-to-date educational material, blending Information and Communication Technologies (ICT) in teaching and learning, and by revising the assessment systems (EU, 2020a). In addition, another strategic objective was to strengthen and upgrade Cyprus' higher education institutions (EU, 2018c). These measures have resulted in a drop in the early school leaving rate and in significant improvements in the attainment levels, in tertiary education, in recent years.

Yet, EU (2020a) reported that a third of Cypriot graduates were employed in occupations that do not require tertiary education. This figure has remained stable over the last decade, thereby indicating a significant challenge in terms of matching the Cypriot graduates' skills with the requirements of Cyprus' labour market. EU (2022a) indicated that Cyprus is also underperforming when it comes to gender equality. It noted that there were less females who pursued tertiary education and who were gainfully occupied in full time employment. This issue could have triggered by their caring responsibilities (of young children). Notwithstanding, the island is experiencing a low participation in vocational education and adult learning. Many young Cypriot adults are not in education, training or in employment, and often lack digital and transferable skills (EU, 2022a).

The students with a migrant background were more likely to be underachievers than native students. Other differences were noticed between disadvantaged and advantaged schools, as private schools were outperforming public schools by more than one year of schooling. Cypriot authorities were taking remedial measures to improve the quality of their education institutions. They introduced migrant integration policies and enacted legislation to foster inclusive education. However, Cyprus still needs to articulate integration policies that are focused on the post-secondary and/or vocational education and training (VET) of young migrant adults (who are mostly asylum seekers). These reforms can help Cyprus to achieve the EU Commission's objectives on "Education and Training" and to align their provision of education with the labour market requirements (EU, 2020a, 2022a).

The Cypriot government's intention is to address the skill gaps through an increased focus on vocational education and training to support the demands of the labour market. Cypriot education authorities are tracking their VET graduates on placement schemes and are committed to forge strong relationships with business and industry stakeholders on curriculum development (EU, 2018a).

The Cyprus' National Strategy for Youth (2017-2019) implemented new programmes to

a support creativity, innovation and entrepreneurship (among young people);
b disseminate information about education and training opportunities among young people;
c reduce the young adults and adolescents' dependencies on other family members;
d support and empower students;
e encourage them to engage in volunteering activities.

Some of these programmes include Makerspace, Students in Action, Summer Youth Leadership School, Youth Business Development Centres and Youth Guarantee, among others.

The strategic plan (2019-2021) of MoEC comprises eight strategic aims that were intended to improve the delivery of quality education and the provision of training to human resources, in the realms of education (EU, 2018c). Hence, the Cypriot government has invested in its educators. It developed a "professional development" framework that specified the training requirements of each school. Moreover, it modified the administrative structures of the Cypriot educational system in order to improve the quality of education services.

A number of different policies such as the establishment of evening technical schools and the new apprenticeship system in Cyprus have resulted in the strengthening of the vocational education and training (VET), however the participation levels and VET graduate employability remain low (EU, 2018a, 2020a). The Cypriot government is making efforts to attract students to VET and lifelong learning. It is establishing relationships with employers from different businesses and industries to provide apprenticeship opportunities to prospective VET students (EU, 2020a).

Despite these ongoing reforms in education, the Cypriot students had low performance levels in digital, science, technology, engineering and mathematics skills. EU (2022a) reported that Cyprus is still lagging behind in its digital transition in terms of 'the provision of high-capacity network coverage', 'basic digital skills', 'shortages of information communications technology specialists' and 're-

latively high broadband price', when compared to its EU counterparts.

2.2.3 Cyprus' active labour market policies

Following the 2012-2013 financial crisis, Cyprus had registered a significant increase in employment figures, year after year. In 2014, Cyprus introduced a Guaranteed Minimum Income (GMI) scheme that was intended to incentivise work among the most vulnerable individuals in society. This scheme encouraged low skilled or unskilled individuals to participate in active labour market programmes. Hence, it was considered as a good instrument to fight poverty and social exclusion (EU, 2018a). Efforts were made also to improve adult and life-long learning. Since 2012, Cyprus has been implementing projects to promote the European Agenda for Adult Learning. For the years 2017-2019, the Cypriot government has opened evening high schools and evening technical schools that were intended to enhance the knowledge and skills (including digital skills) of adult learners, to improve their employability prospects.

At the same time, Cyprus run an Electronic Platform for Adult Learning in Europe (EPALE) that was aimed at adult educators and trainers. In addition, a project that was co-funded by the European Social Fund established mechanisms for the validation of non-formal and informal learning. Moreover, other schemes from the Human Resource Development Authority of Cyprus (HRDA) were targeted at unemployed individuals and new market entrants.

These initiatives have supported vulnerable individuals and assisted them to find jobs in the services sectors as well as in the construction industry, thereby reducing long-term and youth unemployment figures (EU, 2018a, 2020a). EU (2020a) reported that temporary employment has started to decrease as more employees have been offered permanent positions. This positive development translated to significant salary and wage increases for those individuals who were offered indefinite employment contracts. As a result, their conditions of employment were also ameliorated.

Although unemployment increased slightly during the pandemic, it fell again in 2021 (EU, 2022a). However, Cyprus' labour market faces new challenges for upskilling and re-skilling of employees. Most employees, particularly the older ones, need to improve their digital skills. Notwithstanding, there are a number of young people as well as women, who are still not in employment, education or training (EU, 2020a, 2022a). This is probably caused by certain difficulties in school-to-work transition. Alternatively, the young employees are not declaring their employment. EU (2022b) noted that Cyprus has implemented various VET schemes and other courses that were financed by both national and EU funds. These schemes were aimed at helping vulnerable individuals, including youths, migrants, older adults and people with special needs to train themselves, to increase their chances to return to work.

2.3 Malta

EU (2020b) as well as EU (2022b) reported that Malta has been experiencing fast growth and sustained employment creation. The small island registered fiscal surpluses in recent years before COVID-19. The EU's Social Scoreboards frequently appraise Malta's growth in employment. They indicate that the country's unemployment rates are well below the EU average, during the past few years. Before the emergence of Russia-Ukraine war, the inflation was projected to stabilise at 1.5%. Recently, EU (2022b) suggested that the labour market was performing relatively well, in a post COVID-19 context, but the low participation of women and of other underrepresented groups were affecting labour shortages and their social cohesion.

2.3.1 Maltese education policies

The EU's latest country reports indicated that Malta has several long-term structural challenges including the fiscal sustainability implications of ageing as well as the low skill sets of its older citizens, among other issues. They noticed that the small country's demographic and economic growth are expected to put further pressure on its extant infrastructure and natural resources.

EU (2020b) revealed that some population groups were facing a higher risk of poverty than others. EU (2022b) reconfirmed that Malta still had high levels of early school leaving as well as poor educational outcomes, when compared to other EU nations. The report posited that those children from socially disadvantaged families (e.g. whose parents were single, foreign or with low-income streams) were at higher risk of poverty than other children from middle class and affluent families. The former individuals were less likely to benefit from the best education opportunities on the island and were more likely to lag behind their more advantaged peers. They will usually seek employment after they have completed their secondary education EU (2020b, 2022b).

The Maltese Ministry for Education and Employment (MEE) has drafted coherent strategies to reduce the number of early school leavers and to enhance the lifelong learning opportunities to adults (MEE, 2014a, 2014b). The framework for the (Maltese) Education Strategy for 2014-2024 four goals are to:

i. Improve the educational outcomes of boys and girls in literacy, numeracy, and science and technology competence.
ii. Support educational achievement of children at-risk-of-poverty and from low socio-economic status, whilst reducing the relatively high incidence of early school-leavers.
iii. Increase participation in lifelong learning, and
iv. Raise levels of student retainment and attainment in further, vocational, and tertiary education and training.

MEE (2014a) articulated the Strategic Pillars for policy development, that comprised;

i. The Governance of Education Organisations,
ii. The Social Dimension,
iii. International Dimension,
iv. The Provision of Quality Education,
v. The Student Focus, and
vi. Strategic Innovation.

In 2013, MEE launched an 'Early School Leaving Strategy' which was aimed to reduce the number of students who leave school at an early age, and to motivate them to continue their studies at tertiary levels. However, the Maltese early school leaving rate is still significantly above the EU's average, and has remained almost unchanged since 2017 (EU, 2020b, 2022b). This rate is considerably higher for males than for females.
Nevertheless, Malta has (and is) intensifying its outreach with young adults and adolescents (Camilleri, 2020). It is targeting those individuals who leave school with few skills and competences. OECD's PISA indicated that the Maltese students' participation in VET was much lower than the EU's average (OECD, 2018). The smallest EU country has introduced preventative measures against student dropouts from the education system. Malta implemented the 'National Curriculum Framework'; increased VET opportunities in compulsory education; strengthened the existent 'Validation of Informal' and 'Non-formal Learning' and developed new forms of teaching and learning, such as 'e-Learning' (EU, 2018b).
As a result, the employment rate of VET students, was one of the highest within the EU (EU, 2018b, 2020b). EU (2022b) noted that more Maltese students are pursuing tertiary education, and that they had higher chances than their EU counterparts to find employment when graduating.

2.3.2 Maltese active labour market policies

EU (2020b) indicated that the employment rate has reached 75.5% in 2018. Recently, the country has recorded one of the highest employment growths within the EU (EU, 2022b). Moreover, the unemployment rate among young people and long term unemployed, was at a record low.
Evidently, the Maltese authorities were supporting low-skilled individuals, including youths, to improve their employability prospects. The Jobs Plus, formerly known as Employment and Training Corporation (ETC) has made good use of the European Social Funds (ESF) to address the challenge of skill gaps and mismatches in the labour market (ESF, 2009; EU, 2013b, 2020b). The Maltese government relied on ESF funds to create occupational opportunities for disadvantaged individuals and households which were at risk of poverty. It opened social welfare offices called LEAP centres, in different locations around the island, to provide employment and education opportunities to vulnerable groups in society including to single parents, people with disabilities, ex-offenders, migrants and the working poor, amongst others. These segments are considered vulnerable or disadvantaged when compared to other citizens. Hence, the LEAP programmes target inactive, jobless individuals. They are intended to facilitate their access to employment.
Malta's active labour market policies include in-work benefits, tax credits as well as benefit tapering for prospective employees who were never in employment. Other initiatives focused on long-term, unemployed women. They comprise attractive income-tax arrangements for women who return to work after pregnancy; increases in maternity and adoption leave; and exemptions from means-testing for income earned by women working on a part-time basis (EU, 2020b).
Individuals, including single persons, women and persons with special needs are encouraged to return to work, through the provision of free childcare centres (EU, 2022b). Despite these efforts, EU (2020b) noted that the activity gap was still high with just 64% of women aged 15-64 were in employment. The gender employment gap remains one of the widest in the EU (EU, 2022b).
For the time being, the Maltese women (like other European women) are more likely to:

i. engage in the labour market on a part-time basis (in 2018, 6.5% of men worked part-time as opposed to 23.0% of women),
ii. fill medium- and low-skilled positions; and
iii. occupy fewer managerial positions than men.

Notwithstanding, the outbreak of COVID-19 has impacted their participation in the labour market, as well as the provision of childcare services for their young children (EU, 2022b). Evidently, the pandemic has reversed the positive trend that was experienced in the previous

years.

The weak labour-market outcomes of women in employment or of individuals with special needs may be explained by their low level of qualifications and educational attainment (despite recent improvements). The Maltese government mandated companies with a staff count of 20 or more employees, to have at least 2% of their workforce composed by persons with a disability. It introduced schemes that supported this transition (as it included subsidies to employers and exemptions from social security contributions). The employers who fail to adhere to this Maltese legislation are requested to make an annual payment (for every person with disability they should be employing) (Plus, 2020). This policy led Malta to improve its disability employment gap. Currently, this metric is above the EU average (EU, 2022b).

EU (2020b) noted that the Maltese share of low-qualified adults was one of the highest within the EU, at the time. The uptake of upskilling and re-skilling schemes remained low, particularly among small businesses (EU, 2020b). The adult participation in education and training stood at 10.8% in 2018, almost as much as the EU's average (11.1%). However, only 4.1% of low-skilled adults participated in training in 2018, despite their greater need for upskilling. Notwithstanding, the labour-market participation of older individuals (who were between 55-84 years of age) remained relatively low at 50.2%, when compared with the EU average (58.7%), even though Malta was (and is currently) facing labour shortages at all skills levels.

EU (2022b) reconfirmed that Malta had one of the highest shares of low-skilled adults. The labour market participation of people over 55, although increasing, is still low. In response to those challenges, the European Social Fund (ESF) has introduced supporting measures to strengthen the provision of active labour market policies, with a special focus on vulnerable people.

Table 1 features an excerpt of the findings from the EU country reports of Cyprus and Malta. It sheds light on their progress, over the last two years, regarding their implementation of social and economic measures relating to education, social cohesion and employment.

3 Discussion

This research provided a descriptive overview of the policy initiatives that can have an impact on the socio-economic development of Cyprus and Malta. It synthesised the findings from the latest EU country reports that shed light on these countries' education, social welfare and employment policies. Both island states are striving in their endeavours to improve social cohesion and their economic growth prospects through the implementation of inclusive education and active labour market policies. Before the outbreak of COVID-19 pandemic, they were moving in the right direction as they were responding to the EU's recommendations, year after year. This research suggests that they were increasingly delivering quality education and training opportunities to their citizens and addressing the skill gaps and mismatches in their respective labour markets. At the same time, these countries' employment rates were rising, and their jobless figures were decreasing.

Cyprus and Malta were taking steps to reduce their early school leaving rates and the number of youths who are not in education and employment (EU, 2020a, 2020b, 2022a, 2022b). Both countries' governments were incentivising the most vulnerable groups in society to join the labour market. They introduced certain measures including the provision of VET to unemployed individuals, as well as continuous professional development and upskilling opportunities to employees in shrinking economic sectors.

Generally, Cyprus and Malta have registered important advances in terms of their countries socio-economic metrics, over these last few years. Both island states have minimised the number of citizens who were at risk of poverty. EU (2020a) posited that Cyprus should monitor those youth who are not in education and employment, the gender employment gap, as well as its unemployment rates. It recommended that Cyprus ought to continue improving the level of the digital skills of its citizens. EU (2020b) clearly indicated that Malta should reduce the numbers of early school leavers. Furthermore, it should continue executing its gender employment policies. EU (2022a) and EU (2022b) suggested that Cyprus and Malta have not resolved these issues, as yet. They reported that there is scope for both Southern European countries to continue developing policy initiatives to improve the social inclusion of vulnerable groups in society, by providing them ongoing education, lifelong learning and training opportunities, as well as with decent job prospects in the labour market.

4 Conclusion and Implications

Relevant academic literature suggest that the provision of quality education and active labour market policies could reduce social inequality among different demographic groups including women, young adults, immigrants, disabled individuals and older workers (Camilleri et al., 2016; Deacon, 2018; Gravani et al., 2019; Gupta et al., 2016; Vladimirova et al., 2016). This research confirms that cohesive and inclusive societies offer numerous opportunities for the upward mobility of disadvantaged

Socio-economic metric		Cyprus 2020	Malta 2020	Cyprus 2022	Malta 2022
Equal opportunities and access to the labour market	Early school leavers from education and training (who are between 18 and 24 years of age)	Average	Critical	To watch	Weak but improving
	Individuals' level of digital skills (who are between 16 and 74 years of age)	Weak but improving	Better than EU average	To watch	Average
	Youth not in education, employment or training (who are between 15 and 29 years of age)	Weak but improving	Better than EU average	To watch	Better than EU average
	Gender employment gap	To watch	Weak but improving	To watch	Weak but improving
Dynamic labour markets	Employment rate	Better than EU average	Better than EU average	Average	Better than EU average
	Unemployment rate	Weak but improving	Better than EU average	Average	Best performer
	Long term unemployment	Better than EU average	Better than EU average	Average	Better than EU average
	Gross disposable household income	Critical situation	N/A	Critical situation	Better than EU average
Social protection and inclusion	Citizens who are at risk of poverty or social inclusion	Average	Better than EU average	Better than EU average	Average
	Children who are at risk of poverty or social inclusion	N/A	N/A	Average	Average
	Impact of social transfers (other than pensions) on poverty reduction	Average	On average	Average	Critical situation
	Disability employment gap	N/A	N/A	Average	To watch
	Housing cost overburden	N/A	N/A	Better than EU average	Better than EU average
	Children aged less than 3 years in formal childcare	Average	To watch	To watch	Average
	Self-reported unmet need for medical care	Average	Better than EU average	Better than EU average	Better than EU average

Table 1: European country reports' social scoreboards of Cyprus and Malta

Source: EU (2020a, 2020b, 2022a, 2022b).

segments in society. The Cypriot and Maltese socio-economic policies are investing in their human capital to improve the well-being of their citizens, and of their national economies. These Southern European states are implementing initiatives that foster a cohesive labour market to reduce the disparities in their societies. At the same time, they are protecting vulnerable individuals by fighting their social exclusion and marginalisation.

This contribution raises awareness on the importance of delivering an inclusive, quality education for all, to improve the countries' socio-economic performance. Arguably, an indispensable requirement for social cohesion is the eradication of poverty, in all of its forms and dimensions. The pursuit towards continuous improvements in compulsory, vocational and higher education can enhance the individuals' social mobility prospects and may increase their quality of life. The ongoing reforms in education ought to be founded on social inclusion and equity principles, as well as on student-centred curricula and learning outcomes. Moreover, the provision of quality education ought to be supplemented with active labour market policies, including initiatives like; in-work benefits, tax rebates, and free childcare facilities, among other measures, to support individuals to pursue their studies or to return in employment. Active employment policies are required to help job seekers to find employment and/or to assist employed individuals to advance in their career ladder, through life-long learning opportunities. This research implies that governments and employers ought to support the most vulnerable groups in society, including single parents, migrants, older adults, long term unemployed and persons with special needs, who would otherwise risk social exclusion.

COVID-19 situation has had a devastating effect on societal wellbeing and the economy at large. Hence, there is scope for academia to use different methodologies and sampling frames to investigate further the impact of this pandemic on the individuals' quality of life, including on their education and employment prospects, in different contexts.

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Review Article

Cannabinoids For Fibromyalgia: An Updated Systematic Review

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Abstract. Fibromyalgia is an increasingly prevalent condition resulting in high morbidity and economic burden for sufferers. Minimal to modest benefit has been achieved by pharmacotherapies, creating a strong rationale for novel therapies. Substantial evidence has implicated the endocannabinoid system in the modulation of fibromyalgia symptoms. However, the therapeutic potential and potential adverse effects of cannabis-based therapy in fibromyalgia are still under-reported, leading to clinicians' hesitation to opt for such therapy. This systematic review examined the literature and provided a critical review of the safety and efficacy of cannabis-based therapy in fibromyalgia. It resulted that medical cannabis is a safe and effective treatment option for fibromyalgia, whilst further research in this area is needed.

Keywords: fibromyalgia, chronic pain, cannabis, cannabinoids, pharmacotherapy

1 Introduction

Fibromyalgia is a relatively common disorder whose primary manifestation is chronic widespread pain (Bair et al., 2020). Prevalence has increased dramatically with the development of more sensitive diagnostic criteria throughout the years, with a strong preponderance being seen in women, although with the most recent modified the American College of Rheumatology (ACR) criteria, numbers in men are also strong on the increase (Jones et al., 2015).

Fibromyalgia carries a significant economic burden, not only because of the functional impairments to the patient's work and domestic life but also due to the cost of health services. Indeed, the yearly cost of these health services is for example about a thousand euros as revealed by a Dutch study (Boonen et al., 2005) Prior pain conditions are strongly associated with fibromyalgia, possibly due to activation of secondary central sensitization (Clauw, 2015). Indeed, patients with fibromyalgia tend to also suffer from conditions such as chronic low back pain, irritable bowel syndrome, temporomandibular disorders, as well as sleep disorders, anxiety and depression (Slade et al., 2020)

The overall approach to treating fibromyalgia is focused on maintaining or improving function, improving quality of life, and managing symptoms (Bair et al., 2020; Løge-Hagen et al., 2019). The mainstay of treatment has so far been focused on non-pharmacologic areas such as patient education, maintenance of sleep hygiene, a balanced diet, regular physical activity and an overall healthy lifestyle. Psychological treatments such as cognitive behavior therapy (CBT) have shown modest results in improving mood, alleviating pain and improving disability (Bernardy et al., 2013).

Pharmacologic therapies have so far shown minimal to modest benefit in fibromyalgia patients. Simple analgesics such as acetaminophen and non-steroidals have not been found effective (Derry et al., 2017). Whilst gabapentinoids and tramadol have been used by clinicians to some reported effect, a Cochrane review of studies concludes that there is inconclusive evidence for gabapentin reducing pain in fibromyalgia (Cooper et al., 2017). Opioids other than tramadol (due to its serotonergic and noradrenergic uptake properties) have not been found effective in fibromyalgia, despite clinicians' ongoing use of these drugs in practice (Goldenberg et al., 2016). Duloxetine, a selective serotonin and noradrenaline reuptake inhibitor (SSNRI) antidepressant, has been shown to improve symptom scores for pain and fatigue (but not insomnia) compared to placebo, however, adverse effects resulted in high dropout rates (Häuser et al., 2013).

In light of all these efforts, there is a strong rationale sur-

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face for the investigation of novel pharmacologic therapies which can be added to the clinicians' arsenal.

The use of the plant Cannabis Sativa has been linked with medicinal and recreational purposes for millennia. Since the discovery and characterization of the endogenous cannabinoid system, several studies have evaluated how cannabinoid compounds and, particularly, how the modulation of the endocannabinoid (eCB) system influences a wide range of functions, from metabolic to mental disorders (Bahji et al., 2020; Blessing et al., 2015; Bonaccorso et al., 2019; Breuer et al., 2016; Campos et al., 2013; Crippa et al., 2011; Faraji et al., 2017; Hill et al., 2009; Korem et al., 2016; Linares et al., 2019; Lisboa et al., 2017; Rosenberg et al., 2017; Scarante et al., 2017; Schier et al., 2012; Yohn et al., 2017). It was demonstrated in the 1980s that the effects of cannabinoids were mediated by their interaction with specific sites, resulting in the activation of a G protein signaling and the inhibition of adenylate cyclase activity (Howlett, 1987). The eCB neuromodulator system regulates emotional, cognitive, neurovegetative and motivational processes (Colangeli et al., 2021).

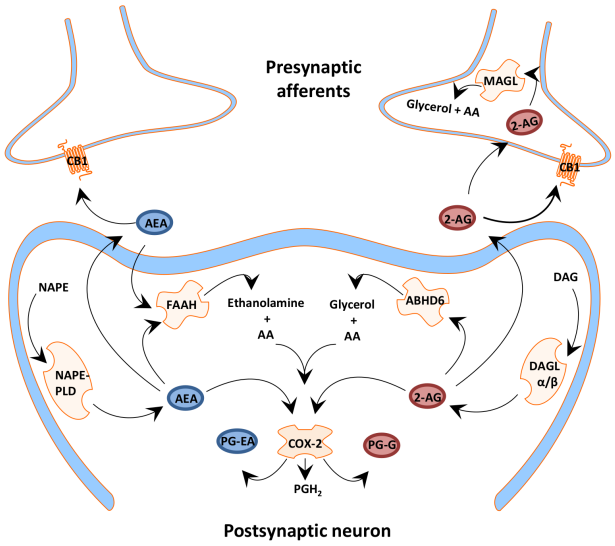

Figure 1: Main pathways of biosynthesis and degradation of two endocannabinoids, anandamide and 2-arachidonyolglycerol. For clarity, only the most investigated pathway for the synthesis of AEA is shown, while possible alternative pathways for AEA formation are not shown, but are described in the text. Abbreviations: CB1, cannabinoid type 1 receptor; AEA, N-arachidonoylethanolamine (anandamide); 2-AG, 2-arachidonylglycerol; NAPE, N-acyl-phosphatidylethanolamine; NAPE-PLD, NAPE-specific phospholipase D; DAG, diacylglycerol; DAGL, DAG-lipase; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; ABHD6, α/β-hydrolase domain 6; COX-2, cyclooxygenase-2; AA, arachidonic acid; PG-EA, prostaglandin-ethanolamide; PG-G, prostaglandin-glycerol ester; PGH2, prostaglandin H2. Taken from Colangeli et al. (2021).

Crucial to the functioning of the eCB system are the cannabinoid 1 and 2 receptors (CB1 and CB2Rs), which have been found in high density throughout various areas of the brain, especially the prefrontal cortex and hippocampus (Zou et al., 2018). Substantial evidence has accumulated implicating a deficit in eCB in the etiology of depression; accordingly, pharmacological augmentation of eCB signaling could be a novel target for the pharmacotherapy of depression (Hill et al., 2009).

Whilst some studies suggest a role for eCB deficiency in pain and specifically, in fibromyalgia the role of endogenous cannabinoids remains unclear and more studies are required in this regard. This is due to the complexity of both the eCB system and pain, a "multidimensional, dynamic integration among physiological, psychological, and social factors that reciprocally influence one another" (Meints et al., 2018). Of note, elevated eCB lipids such as 2-arachidonylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide; AEA) correlated positively with fibromyalgia duration and anxiety & depression respectively (Stensson et al., 2018).

Preclinical data are limited, however, studies indicate reciprocal changes in CB1 and transient receptor potential vanilloid 1 (TRPV1) Rs affect visceral hyperalgesia in chronic stress and fibromyalgia models. Cannabinoids may attenuate low-grade inflammation, another postulate for pathogenesis in people with fibromyalgia (Üçeyler et al., 2011). Cannabinoids may reduce peripheral and central sensitization of nociception by altering cognitive and autonomic processing of chronic pain (Guindon et al., 2009). The distribution of CBRs in the frontal-limbic seems to point toward cannabinoids preferential targeting the affective qualities of pain, known to have an important contribution to the suffering of patients with fibromyalgia (Lee et al., 2012). Finally, considering that fibromyalgia is a stress-related disorder (Van Houdenhove et al., 2004), cannabinoids might help buffer stress and modulate emotional and cognitive functions (Colangeli et al., 2021). Apart from phytocannabinoids, boosting eCBs such as AEA and 2-AG by blocking the specific catabolic enzymes i.e., fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), is being tested as a novel treatment for pain (Papa et al., 2022). Therefore, taking into consideration the complexity of symptom expression and the absence of ideal treatment, the potential for manipulation of the cannabinoid system as a therapeutic modality is attractive. Future research into the clinical utility of eCB metabolism manipulation in fibromyalgia is also expected. With the aim of analyzing the current evidence on the use of cannabinoids in fibromyalgia we, therefore, have run a systematic review of the clinical study.

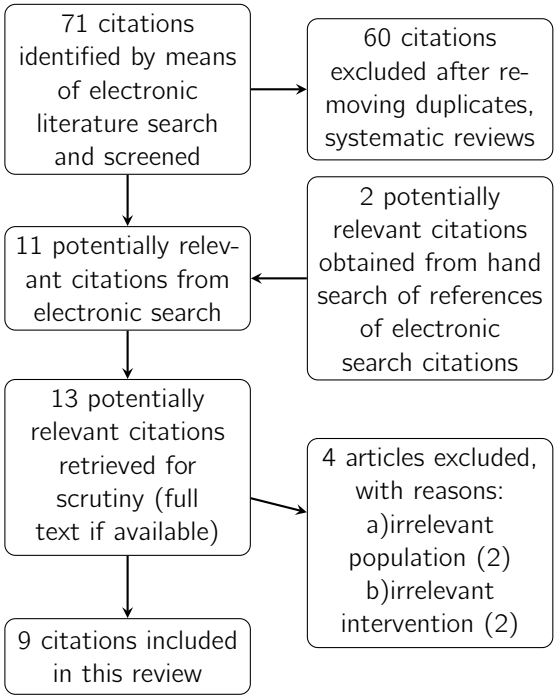

Figure 2: Study flow diagram.

2 Search Methods and Criteria

The PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) guidelines were followed for writing this review. Medline was identified as the database for the research. A search was conducted in March 2022 using the keywords "cannabis + fibromyalgia" and "cannabinoids + fibromyalgia". The following inclusion criteria were used in the selection of studies: a) existing translation in the English Language b) published from 2008 to 2022 c) Randomized Controlled Trials, observational studies, retrospective studies and comparative studies d) minimum of 15 patients in each study e) any preparation of synthetic cannabinoid or herbal cannabis.

3 Results

The search resulted in 31 and 33 results on PubMed for each search term pair respectively, but after removal of duplicates and studies which did not meet study criteria, eight articles were deemed relevant. One of the studies used medical cannabis as an adjunctive therapy to mainstream therapy (see table 1). The references of these studies were also reviewed for additional studies, and one further study was found in this way. The aim was to provide an in-depth screen of the current literature surrounding the action of cannabis and cannabinoid therapies in the outcomes of Fibromyalgia patients, whilst only including studies with validated and reliable methods of data collection, and avoiding anecdotal studies. Table 1 shows a summary of the selected studies and the criteria used to provide this analysis.

4 Dicussion and Critical Analysis

This review included seven studies using herbal cannabis preparations (phytocannabinoids) (Chaves et al., 2020; Fiz et al., 2011; Giorgi et al., 2020; Habib et al., 2018; Sagy et al., 2019; Van de Donk et al., 2019; Yassin et al., 2019) and two studies using the synthetic cannabinoid, nabilone (Skrabek et al., 2008; Ware et al., 2010) in patients with fibromyalgia. The phytocannabinoid preparations were a variety of THC-dominant cannabis flowers and oil extracts.

From the outset, it becomes evident that the reliability of studies using herbal cannabis suffered because of the lack of consistency of cannabis used. In all but one of the studies (Van de Donk et al., 2019), we were unable to identify the strain, potency, cannabinoid profile and terpene profile within the formulation. Only three studies using herbal cannabis included formulations with cannabidiol (CBD) content (Chaves et al., 2020; Giorgi et al., 2020; Van de Donk et al., 2019), and only two included CBD in significant amounts (1:1 ratio with THC) (Giorgi et al., 2020; Van de Donk et al., 2019).

4.1 The dosage varied widely throughout the studies

These problems were not encountered in the two studies using the synthetic delta-9 THC analogue Nabilone (Skrabek et al., 2008; Ware et al., 2010). The re-emergence of plant medicine in the 21st century has so far not been met with sufficient innovations in clinical research attempting to gain quantitative insight into the benefits of this section of medicine. Pharmaceutical markets are now embracing the change of consumer mentality surrounding the ingestion of lab-made synthetic compounds towards more natural products. This shift must not be simply a question of marketing aesthetics, but also viable data.

4.2 Baseline demographics/comorbidities

There was a wide variety of patient characteristics throughout all studies. Most studies showed a prevalence of women, with (Chaves et al., 2020) having a patient cohort consisting of only women. The most abundant cohort by far was women aged 30 to 50 years (Chaves et al., 2020; Fiz et al., 2011; Giorgi et al., 2020; Habib et al., 2018; Sagy et al., 2019; Skrabek et al., 2008; Van de Donk et al., 2019; Ware et al., 2010; Yassin et al., 2019). Women with fibromyalgia are notably younger than men

Main Author(s)	Type of Study	Number of Participants	Duration of Assessment	Type of Cannabinoid Agent Used	Dose	ROA	Adverse Events Reported	Treatment Outcomes
Skrabek et al. (2008)	Randomized, Double-Blind Placebo Controlled Clinical Trial	40	4 weeks	Nabilone, Synthetic cannabinoid	0.5mgOD- 1mg BD	Ingestion	Drowsiness, dry mouth	Statistically significant improvements in VAS, FIQ and anxiety scores when compared to placebo after 4 weeks
Ware et al. (2010)	Randomized, Double Blind,active-control, equivalency crossover clinical trial	31	2 weeks	Nabilone, Synthetic cannabinoid	0.5mg OD 0 1mg OD	Ingestion	Drowsiness	Pain alleviation, improved QoL, improved mood comparable to Amitriptyline. Improved sleep superiorly to Amitriptyline
Fiz et al. (2011)	Observational Cross sectional Study	28	2 hours	THC dominant flower unspecified strain, unofficial sources	Varied, unreliable	Inhalation (smoking), ingestion, combined	Drowsiness, dry mouth	Strong relief for pain, sleep disturbance, stiffness, mood disorders and anxiety. Mild relief for headaches
Habib et al. (2018)	Retrospective Online Self Report Survery	26	N/A	THC dominant flower and oil extract, unspecified strain	17.7-34.6g per month	Inhalation (smoking) 58% vaporisation (23%), conbined (14%), ingestion (8%)	Dry mouth (27%), red eyes(27%) and hunger (15%)	Increased capacity for work in 46% of participants
Van de Donk et al. (2019)	Randomized, placebo Controlled 4-way crossover clinical trial	20	3 hours	Bedrocan, Bedrolite, Bediol medical cannabis flowers, Placebo cannabis	22.4mg Bedrocan, 18.4mg, Bedrolite, 13.4mg Bediol STAT	Inhalation (vaporisation) 100%	Drug high (80%) for Bedrocan, coughing (70%),nausea and dizziness (15%) and sore throat (10%)	THC correlate with increased pain threshold. Bediol resulted in 30% in pain scores
Sagy et al. (2019)	Prospective, Observational Study	367	6 months	THC dominant flower, unspecified strain	670mg-1000mg daily	Inhalation (smoking) & ingestion	Mild dizziness 7.9%), Dry mouth (6.7%) GI symptoms (5.4%)	Reducedpain (44%), Better sleep (73.4%), Depression scores improves (80.8%)
Yassin et al. (2019)	Observational, Cross-Over Study	31	3–6 months	THC dominant flower, unspecified strain	20g monthly	Inhalation (smoking), Inhalation (vaporisation)	Red eyes (90%), Constipation (50%)	Decreased pain intensity, Increased ROM
Giorgi et al. (2020)	Prospective Observational Study	102	6 months	Bedrocan and Bediol		Drops	Dizziness (21%), sleepiness (16%), palpitations (12%), nausea (9%) and dry mouth(9%)	
Chaves et al. (2020)	Randomized, Double-Blind, Placebo- Controlled Clinical Trial	17	8 weeks	THC dominant /trace CBD oil	1.2mg THC/ 0.02mg CBD to 4.4mg THC/0.08mg CBD daily	Ingestion	Somnolence	Improved QoL,pain reduction, improved functionality, improved sense of well being

Table 1

and are more likely to suffer from comorbid psychiatric or connective tissue disorders, whilst men often had multiple medical comorbidities (Sagy et al., 2019). Fibromyalgia shows a gender-dependent, multi-modality of symptoms, and this should be considered in the treatment approach (Arout et al., 2018). Of the studies analyzed in this review, only one study had sufficient recruitment to allow meaningful observations to be made in males (Sagy et al., 2019). The biological mechanisms of interaction of the eCB system differ between sexes, notably how throughout most of the brain, eCB content is estrous cycle-dependent in females in comparison to males, as shown by preclinical studies (Bradshaw et al., 2006). Therefore, the results should not be generalizable between the sexes.
In future studies, findings should be further specified into reference groups according to similar characteristics, both in comorbidities, age and sex demographics.
Post-hoc analysis of the effect of the baseline characteristics on the efficacy of the treatment should be a target for future research endeavors.

4.3 Inclusion/Exclusion Criteria

There were inconsistencies in the inclusion and exclusion criteria between the studies. Some only used fibromyalgia patients with moderate to severe symptoms (Chaves et al., 2020), whilst others excluded patients using any other pain medication than mild analgesics (Van de Donk et al., 2019). One study (Habib et al., 2018) excluded patients with malignancy-associated or rheumatic-associated comorbidities.
Exclusion criteria were more generalizable and frequently included the history of psychotic symptoms or psychosis-associated conditions such as schizophrenia and diagnoses which could explain symptoms other than fibromyalgia.

4.4 Route of Administration

Route of administration of the cannabinoids varied across studies and included smoking, vaporization, ingestion of oil drops or a combination of these. Dosage amount and strategy were also widely varied. There is some evidence in the literature to show that some methods of administration have benefits and risks concerning each other (Aston et al., 2091; Russo, 2016) however there is a lack of studies comparing the benefits and side effect profile of the different routes of administration (ROAs). The general consensus points to vaporization being the safest option, with improved beneficial effects and reduced adverse profiles. This contrasts with smoking, one of the studies (Habib et al., 2018) showed that participants using smoking as their main ROA had a greater incidence and severity of adverse effects such as dry mouth and red eyes. Respiratory problems such as coughing, wheezing and increased phlegm production have been proven to be associated with smoking in various reviews (Peiffer et al., 2018).
It is pivotal for future studies, as well as the future of herbal cannabis preparations in mainstream medicine, that more investigation into ROAs is done. The aim should be the provision of measurable and reliable dose titration, whilst maximizing therapeutic benefit and without causing pulmonary damage.

4.5 Drug Interaction/Comparison with Other Analgesic Treatments

The studies selected did not identify any possible drug interactions or their effects in both the short term and long term. Fibromyalgia patients are often highly comorbid and tend to use many different forms of concurrent analgesia in an attempt to better their symptoms.
Moreover, only one study (Ware et al., 2010) directly compared cannabinoids to mainstream medication options such as amitriptyline, a tricyclic antidepressant, showing the synthetic cannabinoid nabilone to be favorable in both efficacy and side effect profile. It is crucial that in future, studies (including those using phytocannabinoids or herbal cannabis preparations) are compared with other standard treatments for pain management to provide reliable comparison data. This allows an informed discussion over the pros and cons of each treatment in the clinical setting, empowering fibromyalgia patients to make informed choices about their medication as well as providing guidance to medical professionals.

4.6 Diagnostic Validity

Seven of the ninth studies (Chaves et al., 2020; Fiz et al., 2011; Giorgi et al., 2020; Skrabek et al., 2008; Van de Donk et al., 2019; Ware et al., 2010) used established criteria for diagnosis in the recruitment of patients. The criteria used in five of the seven studies were the ACR 2010 criteria. Two studies used the American College of Rheumatology 1990 criteria (Skrabek et al., 2008; Ware et al., 2010). Recommendations for future studies include using recognized criteria for verification of a fibromyalgia diagnosis between physicians. A complicating factor in this context would be that the precise parameters of a fibromyalgia diagnosis are operationalized in an entirely different way between cultures, medical bodies and hospital systems.

4.7 Assessment Outcome

The methods used for the assessment of outcomes in patients varied. Chaves et al. (2020) used the Fibromyalgia Impact Questionnaire (FIQ), an instrument validated for use in this condition (Bennett et al., 2009) to investigate outcomes, whilst Van de Donk et al. (2019) took a completely different approach by comparing spontaneous pain

scores and electrical pain thresholds. Similar to Chaves et al. (2020), Fiz et al. (2011), Giorgi et al. (2020), Habib et al. (2018), Skrabek et al. (2008), Ware et al. (2010) and Yassin et al. (2019) all used validated instruments for assessing the severity of symptoms in fibromyalgia. Moreover, all but Fiz et al. (2011) used a medium-term approach (weeks to months) for the assessment of outcomes. Fiz et al. (2011) assessed short-term outcomes, as did Van de Donk et al. (2019).

The studies using similar instruments for outcome reportage allowed trends to be observed. Some studies explored other experimental designs, this made a meta-analysis of the data less likely to reach definitive conclusions. Future studies need to operationalize the specific type of pain that is being treated for and assessed in fibromyalgia.

It is helpful in the pursuit of reliable clinical data that similar instruments are used to allow cross-referencing and meta-analysis of the results obtained. This allows both studies and reviews to give better peace of mind to clinicians and other professionals using this data to help patients make informed choices about their medication choices.

4.8 Participant Education

It was noted that in the selected studies, little to no focus was placed on participant education. Indeed, it was seen from characterization studies allowing more freedom for patients to choose preferential methodologies of cannabis use, a wide variety of methods were employed by patients to use their medicine. In more organized study designs, no reference was made to teaching patients about the use of the delivery devices and signs of intoxication. Whilst it can be argued that this prevented possible interviewer bias, it is reasonable to assume this had a bearing on results. Patient education is a vital factor in the medical and pharmaceutical industries and will be even more so in the future as the culture of shared decision-making and patient-centric care takes precedence in clinical settings. Patient education should not be limited to naïve users, as even experienced cannabis users tend to derive most of their knowledge from their own experiences, and this knowledge tends to show discrepancies from available evidence (Kruger et al., 2020).

Notably, several participants who dropped out from the initial phases of some studies (Skrabek et al., 2008; Ware et al., 2010) mentioned the stigma surrounding cannabis as a factor. The data from more recent studies do not explore whether stigma continues to be a factor affecting treatment outcomes. This is an important consideration for future studies, especially those exploring pain syndromes and cannabis.

4.9 Participant Blinding

In studies that incorporated blinding of participants, challenges were generally faced by those using THC-dominant herbal cannabis preparations. Indeed, Van de Donk et al. (2019) used Bang's Blinding index (Bang et al., 2004) to show that Bedrocan faced the biggest issues concerning blinding, however, successful blinding was still possible according to the blinding index's cut-off rate (< 0.5). The lack of psychoactive effects with placebo compared to potent THC-dominant strains is a further challenge for clinical trials, possibly leading to an overestimation of the THC-dominant preparations' beneficial and/or adverse effects.

These issues are less problematic in the study of non-psychoactive cannabinoids, which constitute a large majority of the phytocannabinoids found in nature.

4.10 Adverse Effect (baseline, assessment of severity)

In all the nine studies included in this review (Chaves et al., 2020; Fiz et al., 2011; Giorgi et al., 2020; Habib et al., 2018; Sagy et al., 2019; Skrabek et al., 2008; Van de Donk et al., 2019; Ware et al., 2010; Yassin et al., 2019), adverse effects were mild to moderate and the major adverse effects seen were drowsiness and a dry mouth. Other adverse effects reported in a minority of the participants included mild dizziness, red eyes, hunger and palpitations. Studies using smoking as a route of administration also included sore throat and coughing as immediate side effects. This was also seen in other inhalation methods such as vaporization. Nausea was generally observed in ingestible oil preparations.

Van de Donk et al. (2019) was the only study where patients reported a significant drug high, whilst the experience of the psychoactive effects in patients was not explored in detail. This was only seen with a THC dominant strain Bedrocan, and not with CBD dominant strain Bedrolite or Bediol, which contains CBD:THC in a ratio of approximately 1:1. Patients recruited for this study were cannabinoid naïve and instructed to fast before the study. It is unclear if this is significant to the adverse effect findings. Together with the in-depth, jargon-free participant education described above, more research into the psychoactive actions of THC and the milieu of interacting factors is necessary for THC to be incorporated safely into medical use.

Furthermore, future studies should aim to create a baseline for symptoms commonly seen in fibromyalgia, to distinguish them from adverse reactions due to medical cannabis use. For example, symptoms such as dizziness, nausea, constipation, dry eyes and dry mouth are consistent in both (Van de Donk et al., 2019). Additionally,

whilst some studies did include a control group, future studies should control for cannabis use patterns amongst patients, such as naïve patients, past users, current users, and non-users. Ideally, future studies should control for medical and recreational use since the participant backgrounds and motives surrounding cannabis use in these two groups might vary and cause significant interference in the results.

5 Conclusion and Final Recommendations

In conclusion, the current evidence is indicating that cannabinoids have the potential to be a safe and effective treatment modality for patients with fibromyalgia. The significant limitations of the current body of evidence prevent more definitive statements and more widespread application of cannabinoids in a clinical setting. Our conclusions concur with those of other authors looking at pharmacological advances in fibromyalgia syndrome including cannabinoids (Khurshid et al., 2021). The shining potential of this treatment option to provide much-needed, safe effective relief in these patients, together with these limitations, provide a strong rationale for further studies into the subject.

In summary, recommendations for future studies include stratification of findings into reference groups according to similar characteristics, as well as a post-hoc analysis of the effect of the baseline characteristics on the treatment outcomes. Future research should include an analysis of the effect of ROAs, more insight into common and uncommon drug interactions of cannabinoids and the effect of concurrent analgesia. There is a need for more generalizability across studies, and instruments used for outcome reportage should be standardized. Inclusion/exclusion criteria also require more standardization, whilst ensuring that no form or severity of fibromyalgia is excluded, and that established criteria are used for widespread diagnostic validity. Adverse effect profiles need further analysis, with larger sample sizes and classified according to a baseline of the present symptoms of fibromyalgia. More work needs to be done concerning the challenges of effective participant blinding when using cannabinoids in clinical research.

The legal climate surrounding medical and recreational cannabis use is dynamic and changing rapidly, especially in the European Union where we are seeing a swathe of member states regulating the use of cannabinoids in medical, but also cosmetic, recreational, and nutritional contexts. Policymakers would be wise to identify clinical research as a pivotal starting point for these measures and make decisions based on the best evidence. It is vital also for regulators and governments to ensure market concerns do not out-pace research. Finally, patient education and patient attitudes towards cannabinoids, their benefits and risks, should be a backbone of future clinical integration of these substances. Innovations in medical science are moot if not translated, accurately and simply, into a widespread culture of responsible use of medicines in our societies.

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Review Article

Interferometric phase denoising and unwrapping: a literature review

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Abstract. Interferometric SAR (InSAR) phase denoising and phase unwrapping are two key steps of the InSAR pipeline, leading to estimated deformation maps. The objective of this paper is to provide an overview of the recent literature in the field of InSAR phase denoising and unwrapping, and identify the most promising techniques, as well as benchmarks for performance comparison. Summaries of the performance metrics of the various methods are also provided. An example use case of InSAR techniques, including phase denoising and unwrapping, to estimate deformation following a volcanic eruption is provided.

Keywords: InSAR, phase denoising, phase unwrapping

1 Introduction

Freely-available satellite data provides a wealth of regularly updated data which can be used to monitor effects of coastal erosion and provide an early warning system against hazards. As opposed to optical and thermal satellite imagery, Synthetic Aperture Radar (SAR) images are produced by active remote sensing, in which microwaves are beamed from the satellite towards Earth, and the reflected waves are detected by sensors onboard the satellite. Ground displacements of a few millimeters from one time-series image to another can be detected. The advantages of SAR remote sensing is that images can be acquired in any type of weather conditions, day or night.

The Copernicus programme's Sentinel-1 satellite constellation (European Space Agency, 2012) provides C-band SAR capability, with a repeat frequency of 6 days and a revisit frequency of around 2 days over Europe. The data is acquired in four modes: Extra Wide (EW) Swath mode (spatial resolution: 25 m x 100 m), Interferometric Wide (IW) Swath mode (spatial resolution: 5 m x 20 m), Strip Map (SM) mode (spatial resolution: 5 m x 5 m) and Wave (WV) mode (spatial resolution: 5 m x 20 m, mainly used over open ocean). The Stripmap mode is only available for emergency situations and certain select geographical locations (and is not available online for Malta through the Copernicus Open Access Hub (European Space Agency, 2022)). The IW mode is the one typically used for interferometric analysis and land subsidence detection.

An Interferometric SAR (InSAR) image, also known as an interferogram, is created from two temporally separated single look complex (SLC) SAR images via the pixel-wise product of one SLC image with the complex conjugate of the other SLC image. Thus each pixel in an interferogram indicates the phase difference between two co-registered SLC images. The phase difference encodes useful information including deformation of the earth's surface. A differential interferogram is created when an external DEM is used to subtract the topographic information from the interferogram.

At this point, it is vital to denoise the resulting interferogram, as in particular the phase noise will significantly affect all subsequent stages from phase unwrapping to motion signal modelling. The phase noise can be modeled as additive noise. The classical and most widespread denoising approach is to use a multilook filter (Jong-Sen Lee et al., 1994a), which applies a simple moving average on neighbour pixels in a rectangular window, i.e. boxcar filtering. The disadvantages of the multilook filter are the resolution loss and phase fringe distortion when dealing with the high-topography and high-heterogeneity areas. The multilook filter assumes that the interferometric phase is locally stationary and the scene reflectivity is homogeneous in a local window, where the selected samples are independently and identically distributed (i.i.d.). In this case, the multilook filter expects to perform a maximum likelihood (ML) estimation (Seymour et al., 1994), which is also the foundation of most phase filtering methods.

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However, this assumption is not always true due to the topography variation and reflectivity heterogeneity, especially when faced with the scenes of region edge, structure and texture. In this case, the interferometric phase tends to exhibit the characteristics of nonstationarity and nonhomogeneity, conflicting with the i.i.d. assumption.

The accuracy of the phase measurement in the interferogram is limited by the magnitude of the interferometric coherence, which describes the degree of correlation between the two radar images. There are a number of factors which contribute to a reduction of coherence, including receiver noise, temporal and geometric decorrelation. Therefore, the estimated coherence map of an interferogram is a crucial indicator showing the reliability of the interferometric phase.

The produced interferograms consist of a wrapped phase limited to the interval $(-\pi, \pi]$, resulting in phase discontinuities. Phase unwrapping is then computed to obtain the true phase, which is generally considered to be the most complicated stage of InSAR processing. This is however a necessary step in order to obtain height information. Single baseline phase unwrapping is an ill-posed inverse problem, as there are infinite solutions. The SNAPHU plugin for SNAP is a widely used tool to perform 2D phase unwrapping (C. W. Chen et al., 2002). It treats phase unwrapping as a maximum a posteriori probability estimation problem, and tries to compute the most likely unwrapped solution given the data available. The optimization problem is solved approximately using network-flow techniques.

In this paper, we review a number of state-of-the-art methods for InSAR phase denoising and unwrapping, and provide a comparison between the methods in each case using appropriate metrics. In addition, as an example, we show how these techniques can be applied to estimate the deformation that occurred at Mount Etna following an eruption in December 2018.

2 Denoising of SAR interferometric phase

2.1 Methods

There are several works in the literature which focus on, as well as a number of EO processing pipelines which support denoising (or restoration) of interferometric phase. The boxcar filter (Jong-Sen Lee et al., 1994b) is a well-known method, which simply performs a moving average to estimate the variation of the local pixel pattern. However, it results in a loss of spatial resolution, and it is not suitable for areas with large slopes. The Lee filter (Jong-Sen Lee et al., 1998) is another well-known classical method. It takes advantage of the local fringe morphology and reduces the noise via local statistics and an adaptive window. On the other hand, the Goldstein filter is a frequency domain method (Goldstein et al., 1998). As part of its SAR interferometry processing chain, SNAP has inbuilt functionality for denoising after generating an interferogram. This involves performing multilook processing followed by applying a filter (such as the Goldstein filter).

Following the realization that clean signal phase values are also correlated in the temporal domain, in recent years, many methods have started taking the interferogram stack into consideration. Theoretically, it is easier to extract displacement information over a longer period of time. DespecKS (Ferretti et al., 2011) introduced a space adaptive processing together with their SqueeSAR procedure that could filter interferometric phase properly by using amplitude SAR images.

Another modern concept is that of nonlocal filtering, where the idea is to exploit further information from the data itself. In general, images contain repetitive structures such as corners and lines. Those redundant patterns in an image could be analyzed and explored to improve filtering performance. More and more studies are deploying nonlocal techniques for interferometric phase denoising (R. Chen et al., 2013; Deledalle et al., 2011; Zhu et al., 2014). The first nonlocal method applied to interferometric phase filtering was proposed by Deledalle et al., 2009. Both image intensities and interferometric phase information are used to build a nonlocal means model with a probability criterion for estimating pixels. NL-InSAR (Deledalle et al., 2011) is the first InSAR application to use a non-local approach for the joint estimation of the reflectivity, interferometric phase and coherence map from a pair of coregistered SLC SAR images. In (R. Chen et al., 2013) and Lin et al., 2015, researchers achieve better results on textural fine details preservation by combining non-local filtering with other conventional natural image processing algorithms, such as pyramidal representation and singular value decomposition. A unified framework (NL-SAR) is proposed in (Deledalle et al., 2015) as an extension of NL-InSAR, where an adaptive procedure is carried out to handle very high resolution images. It is able to obtain the best nonlocal estimation with good quality on radar structures and discontinuities reconstruction.

Another popular algorithm, nonlocal block-matching 3D (BM3D) which is widely used for additive white Gaussian noise removal for natural images, also inspired researchers to propose InSAR-BM3D (Sica et al., 2018) which delivered state-of-the-art results for InSAR phase filtering. The method is not able to concurrently estimate phase coherence. Instead, InSAR-BM3D requires a coherence map as input and as a result, the performance

is likely affected by the accuracy of the coherence estimator.

Recently, machine learning methods have also demonstrated excellent performance in the task of image restoration and denoising. A number of these techniques have also been applied to the problem of interferometric phase denoising. In (Kang et al., 2021), the authors propose a complex convolutional sparse coding algorithm, which avoids staircase effects and preserves the details of phase variations. A Fully Convolutional Network is used in (Li et al., 2019) to segment layover areas from the normal pixels, and a denoising convolutional neural network to estimate the phase noise and remove it from the interferogram. This method also demonstrates the improvement given by the denoising on the phase unwrapping procedure. A CNN encoder-decoder approach is used in (Mukherjee et al., 2018) to denoise SAR interferograms, with a two-channel input and output consisting of the real and imaginary part of the interferogram.

Residual learning is used to obtain interferometric phase denoising in (Liu et al., 2021). The CNN architecture is based on the Denoising CNN (DnCNN) framework proposed in (K. Zhang et al., 2017), however uses pre-activation instead, in which batch normalization and the activation function (ReLU) are applied before the weight layers, as opposed to the usual post-activation where the activation function is applied at the end. In terms of evaluation metrics, both PSNR and Number of Phase Unwrapping Errors (NoUE) were used.

The DeepInSAR method (Sun et al., 2020) uses a CNN architecture to extract features from a concatenated input of the real and imaginary components of the noisy phase, and the normalized amplitudes of the two SLC acquisitions to extract features, which are then used to perform phase filtering and coherence estimation simultaneously using two further CNN sub-networks. DeepInSAR outperformed the box car filter and the NL-SAR and NL-InSAR methods both in terms of RMSE and SSIM.

A scale recurrent neural network (RNN) is used in (Pu et al., 2020) to achieve interferometric phase filtering, in which RNN units are used to connect three different-scaled subnetworks based on an encoder-decoder architecture. In this way, global structural phase information contained in the different-scaled feature maps can be used. On the same simulated dataset, the overall performance of this method is better than of DeepInSAR (Sun et al., 2020).

A different approach was taken by (Mukherjee et al., 2020), which used an unsupervised generative model to perform joint phase filtering and coherence estimation, which directly learns the InSAR data distribution, i.e. the bivariate (real and imaginary) Gaussian parameters μ and σ for a centre pixel in a given neighbourhood. The method outperforms the NL-SAR, NL-InSAR, as well as the Goldstein and box car filters in terms of RMSE and computational time.

2.2 Metrics

The majority of the methods reviewed focused on RMSE as a performance metric, followed by PSNR and SSIM, which are standard image processing metrics. However, two papers also presented the Number Of Residues (NOR) (Bamler et al., 1998). Residues are points of two-dimensional phase inconsistency determined by integration of the phase differences around closed paths, and therefore it is desirable to minimize them. Another metric occasionally presented is the phase cosine error.

2.3 Comparison of methods

A comparison of some state-of-the-art phase denoising methods is shown in table 1. As each method evaluated the performance on a different dataset, except in one case, and due to the variety of metrics used, the metrics reported in each of the papers when comparing the method to previous techniques is also reported.

3 Phase Unwrapping

3.1 Methods

Phase unwrapping is a crucial signal processing problem in several applications, such as digital holographic interferometry, SAR and Magnetic Resonance Imaging, in which the aim is to restore the original phase from the wrapped phase. As a result, there are several works both in remote sensing journals as well as in signal and image processing journals. In SAR interferometry, almost all single-baseline phase unwrapping methods exploit the phase continuity assumption (also known as the Itoh condition, which requires that the absolute phase difference between any two neighbouring pixels be less than π Yu et al., 2019. However, system noise and abrupt topographic changes or deformation frequently result in the failure of the Itoh condition in practice. The state-of-the-art currently lies in the use of deep learning techniques with convolutional neural networks (Spoorthi et al., 2019; Wang et al., 2019; Zhou et al., 2020).

As a result, most modern techniques focus on multi-baseline phase unwrapping (MB PU), which is a well-posed problem as it can take advantage of baseline diversity to significantly increase the ambiguity intervals of interferometric phases, and completely overcomes the limitation of the Itoh condition. MB PU methods can be divided into two major groups: parametric and nonparametric methods. The former make use of the InSAR pdf to formulate a maximum likelihood (Fornaro et al., 2006;

Method	Year	Dataset	RMSE (radians)	SSIM	NOR	PSNR (dB)
InSAR-BM3D (Sica et al., 2018)	**2018**	**Simulated data[1]**	**0.2858**	-	**282.18**	-
NL-SAR (Deledalle et al., 2015)	-	Simulated data[1]	0.5045	-	996.95	-
NL-InSAR (Deledalle et al., 2011)	-	Simulated data[1]	0.5358	-	523.30	-
Goldstein (Goldstein et al., 1998)	-	Simulated data[1]	0.7233	-	2662.13	-
Lee (Jong-Sen Lee et al., 1998)	-	Simulated data[1]	0.4973	-	377.03	-
BoxCar (Jong-Sen Lee et al., 1994b)	-	Simulated data[1]	0.5113	-	354.45	-
DeepInSAR (Sun et al., 2020)	**2020**	**Simulated data[2]**	**0.9593**	**0.7976**	-	-
BoxCar (Jong-Sen Lee et al., 1994b)	-	Simulated data[2]	1.2096	0.5150	-	-
NL-SAR (Deledalle et al., 2015)	-	Simulated data[2]	1.2801	. 0.4684	-	-
NL-InSAR (Deledalle et al., 2011)	-	Simulated data[2]	1.1890	0.5202	-	-
GenInSAR (Mukherjee et al., 2020)	**2020**	**Simulated data[3]**	**0.687**	-	-	-
CNN-InSAR (Mukherjee et al., 2018)	-	Simulated data[3]	1.270	-	-	-
NL-SAR (Deledalle et al., 2015)	-	Simulated data[3]	1.537	-	-	-
NL-InSAR (Deledalle et al., 2011)	-	Simulated data[3]	0.850	-	-	-
Goldstein (Goldstein et al., 1998)	-	Simulated data[3]	1.260	-	-	-
BoxCar (Jong-Sen Lee et al., 1994b)	-	Simulated data[3]	1.025	-	-	-
SRN (Pu et al., 2020)	**2020**	**Simulated data[4]**	**0.6340**	**0.8811**	**0.004**	-
Lee (Jong-Sen Lee et al., 1998)	-	Simulated data[4]	1.5372	0.2008	369	-
Goldstein (Goldstein et al., 1998)	-	Simulated data[4]	1.2182	0.4617	16	-
InSAR-BM3D (Sica et al., 2018)	-	Simulated data[4]	0.9070	0.7366	0.012	-
SRN (Pu et al., 2020)	-	**same as DeepInSAR**	**0.6703**	**0.8606**	-	-
DeepInSAR (Sun et al., 2020)	-	same as DeepInSAR	0.8536	0.8666	-	-
In-CNN (Liu et al., 2021)	**2021**	**Simulated data[5]**	-	-	-	**39.183**
WFT (Kemao, 2007)	-	Simulated data[5]	-	-	-	36.352
In-BM3D (W. Zhang et al., 2014)	-	Simulated data[5]	-	-	-	34.957
SP (Hongxing et al., 2015)	-	Simulated data[5]	-	-	-	37.019
GS	-	Simulated data[5]	-	-	-	37.082
DnCNN (K. Zhang et al., 2017)	-	Simulated data[5]	-	-	-	35.633
ComCSC-GR (Kang et al., 2021)	**2021**	**Simulated data[6]**	-	-	-	**31.140**
InSAR-BM3D (Sica et al., 2018)	-	Simulated data[6]	-	-	-	30.575
ComCSC (Kang et al., 2021)	-	Simulated data[6]	-	-	-	26.975
NL-SAR (Deledalle et al., 2015)	-	Simulated data[6]	-	-	-	27.830
NL-InSAR (Deledalle et al., 2011)	-	Simulated data[6]	-	-	-	28.658
Goldstein (Goldstein et al., 1998)	-	Simulated data[6]	-	-	-	19.203
BoxCar (Jong-Sen Lee et al., 1994b)	-	Simulated data[6]	-	-	-	24.685

[1] Average performance on cones, peaks, ramps and squares
[2] High AWGN, with low amplitude strips and high fringe frequency level (S2-S-F3)
[3] Gaussian bubbles, roads and buildings
[4] Generated using Gaussian distributed random matrix
[5] Mountains (Gaussian $\sigma = 0.5$)
[6] Average performance on mountains, peaks, shear plane, squares

Table 1: Summary of the performance of recent interferometric phase denoising methods.

Pascazio et al., 2002) or maximum a posteriori framework (Ferraiuolo et al., 2004; Fornaro et al., 2002; Poggi et al., 2000), while the latter make use of unsupervised learning techniques to estimate absolution phase. Typically, clustering algorithms are used to group pixels with the same 2π-ambiguity, such that the cluster centroid can then be used to estimate the terrain heights of all pixels in the same cluster (Yu et al., 2011).

A new processing flow is proposed in (Wu et al., 2020), in which the authors develop two CNNs for fast detection of deformation caused by mining (DDNet) followed by phase unwrapping (PUNet). The training dataset of phase interferograms was developed by simulating the three components (deformation phase, turbulent atmospheric phase and decorrelation noise) independently and then superimposing them together to get the final simulated phase. A distorted 2D Guassian surface was used to simulate the deformation phase, which results in a typical bell shape representing the ground subsidence caused by mining. They compared the performance of the StaMPS (Hooper et al., 2004) method to the PUNet in extracting Persistent Scatterer (PS) points, and found that the PUNet method was able to estimate the maximum subsidence rate much better than the StaMPS method.

The problem of phase unwrapping is tackled from a semantic segmentation point of view in (Sica et al., 2020). A U-Net architecture is used to map the phase interferogram and coherence to the range/azimuth wrap count gradient, which can then be used to derive the unwrapped phase field. The coherence is useful as an additional input feature as it helps the network to identify and manage critical noisy regions. When compared to other methods, such as Statistical-Cost, Network-Flow Algorithm for Phase Unwrapping (SNAPHU) (C. W. Chen et al., 2002) and PU via MAx flows (PUMA) (Bioucas-Dias et al., 2007), it performs better in terms of RMSE. A U-Net architecture is also used in (Z. Zhang et al., 2020) to estimate the number of integer multiples of 2π (ambiguity number) to add to the wrapped phase.

3.2 Metrics

The vast majority of papers make use of the RMSE in order to quantify performance.

3.3 Comparison of methods

A comparison of some state-of-the-art phase unwrapping methods is shown in table 2. As each method evaluated the performance on a different dataset, the metrics reported in each of the papers when comparing the method to previous techniques is also reported.

4 Application of InSAR phase denoising and unwrapping techniques

On 24-27 December 2018, an eruption of Mount Etna took place, resulting from a complex interaction between tectonic and volcanic processes on the volcano's flanks. We have repeated the DInSAR analysis presented in (De Novellis et al., 2019) to show the intermediate stages of the procedure, from the generation of the interferogram to the subsequent filtering, phase unwrapping and finally estimation of displacement.

Two Sentinel-1 SLC acquisitions in descending orbit from the 22nd and the 28th December 2018 respectively were downloaded from the Copernicus hub[1]. Each Sentinel-1 SLC acquisition is divided into three subswaths, and each subswath is made up of 9 bursts. As Mount Etna straddles two subswaths (IW1 and IW2), the SNAP application was used to extract the appropriate bursts, co-register the two acquisitions and generate the interferogram from the phase difference of the two merged subswaths (see figure 1(a)). The flat-earth phase due to the Earth's curvature and the topographic phase contribution are subtracted to produce the final interferogram. Following this, the Goldstein filter was used to denoise the interferogram (see figure 2).

In the interferogram, the phase is wrapped in the range $(-\pi, \pi]$. Therefore, the SNAP application is used to unwrap the phase using the Minimum Cost Flow algorithm (see figure 2(a)). The relation between unwrapped phase (in radians) and displacement (in metres) is given by:

$$d = -\frac{\lambda}{4\pi}\Delta\phi_d \tag{1}$$

where λ is the wavelength of Sentinel-1's C-band SAR, and $\Delta\phi_d$ is the unwrapped phase, is then used to obtain the deformation map shown in figure 2(b). This map shows evidence of up to 25 cm of subsidence on the eastern side, and up to 20 cm of uplifting on the western side.

5 Summary

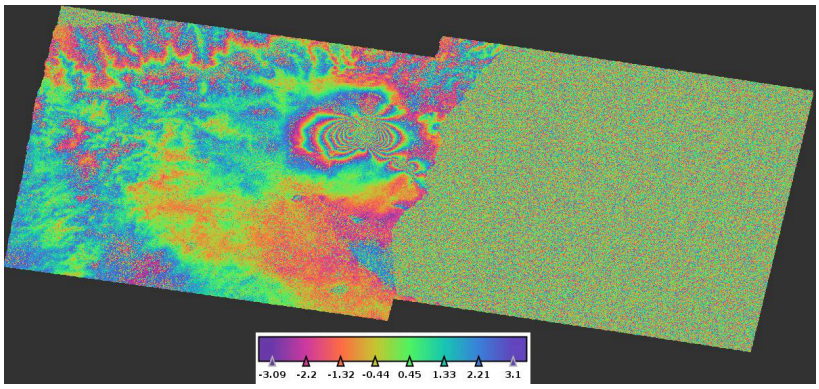
This paper has reviewed the state-of-the-art in SAR interferometric phase denoising and unwrapping. In the Coastal SAGE project, we aim to go beyond the state-of-the-art in interferometric SAR denoising by considering deep learning specifically for the problem of generating noise-reduced interferometric phase. The enhanced quality interferograms will preserve spatial resolution, allowing for more detailed displacement and deformation monitoring using standard PSI techniques. Deep learning architectures will also be used to tackle single-baseline

[1] https://scihub.copernicus.eu

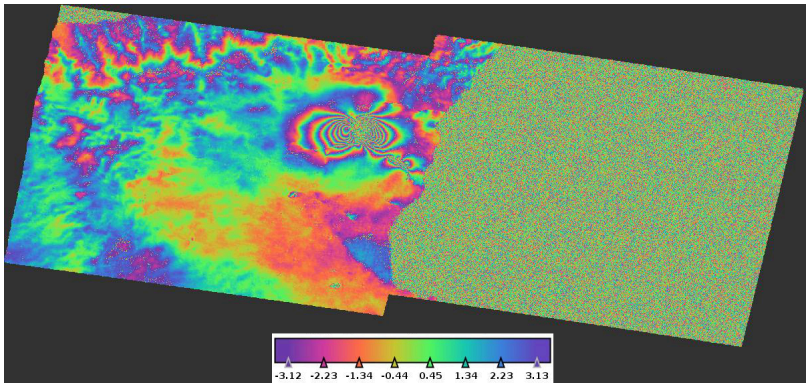
Method	Year	Dataset	RMSE (radians)	Phase Error (radians)
PhaseNet (Spoorthi et al., 2019)	**2019**	**Simulated data[1]**	**1.414**	-
QGPU (Ghiglia et al., 1998)	-	Simulated data[1]	3.317	-
MATLAB's Unwrap (MATLAB, 2022)	-	Simulated data[1]	4.123	-
LPM-TSPA (Lan et al., 2019)	**2019**	**Simulated data[2]**	**1.030**	-
L1-Norm (Costantini, 1998)	-	Simulated data[2]	1.122	-
TSPA (Yu et al., 2016)	-	Simulated data[2]	1.319	-
CNN+ (Sica et al., 2020)	**2020**	**Real data[3]**	**3.90**	-
CNN (Sica et al., 2020)	-	Real data[3]	4.52	-
Branch Cut (Goldstein et al., 1988)	-	Real data[3]	10.05	-
LS (Ghiglia et al., 1994)	-	Real data[3]	8.77	-
PUMA (Bioucas-Dias et al., 2007)	-	Real data[3]	4.62	-
SNAPHU (C. W. Chen et al., 2002)	-	Real data[3]	4.08	-
Region Segmentation (Z. Zhang et al., 2020)	**2020**	**Simulated data[4]**	-	**-0.0113**
Least Squares (Ghiglia et al., 1994)	-	Simulated data[4]	-	0.9035
Network Flow (Costantini, 1998)	-	Simulated data[4]	-	0.3448
Branch Cut (Goldstein et al., 1988)	-	Simulated data[4]	-	0.0524
PGNet-PU (Zhou et al., 2020)	**2020**	**Simulated data[5]**	**0.0181[6]**	-
Branch Cut (Goldstein et al., 1988)	-	Simulated data[5]	0.1981[6]	-
MCF (Costantini, 1998)	-	Simulated data[5]	0.0430[6]	-
SNAPHU (C. W. Chen et al., 2002)	-	Simulated data[5]	0.0400[6]	-
PUMA (Bioucas-Dias et al., 2007)	-	Simulated data[5]	0.0408[6]	-
PGNet-PU (Zhou et al., 2020)	**2020**	**Real data[7]**	**0.0088[6]**	-
Branch Cut (Goldstein et al., 1988)	-	Real data[7]	0.1054[6]	-
MCF (Costantini, 1998)	-	Real data[7]	0.0423[6]	-
SNAPHU (C. W. Chen et al., 2002)	-	Real data[7]	0.0283[6]	-
PUMA (Bioucas-Dias et al., 2007)	-	Real data[7]	0.0107[6]	-

[1] Repeated arithmetic operations (additions and subtractions) on Gaussian functions with randomly varying means and variances; SNR = 0 dB.
[2] Simulated DBInSAR dataset for Isolation Peak region of Colorado; short baseline.
[3] Dataset consists of 10 different patches of 512x512 pixels from a real TanDEM-X single-pass interferogram. For each patch the error is computed between the estimated unwrapped phase field and the reference absolute phase, obtained by back-geocoding the SRTM DEM.
[4] 2D Gaussian distribution with multiple peaks and varying means and variances.
[5] Simulated reference terrain height from the SRTM DEM at Lhasa, Tibet
[6] Normalized RMSE
[7] TerraSAR-X-TanDEM-X interferometry image covering Lhasa, Tibet

Table 2: Summary of the performance of recent phase unwrapping methods.

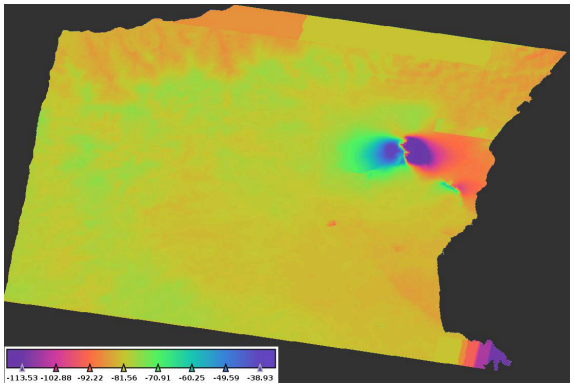


(a) Unfiltered interferogram (radians)

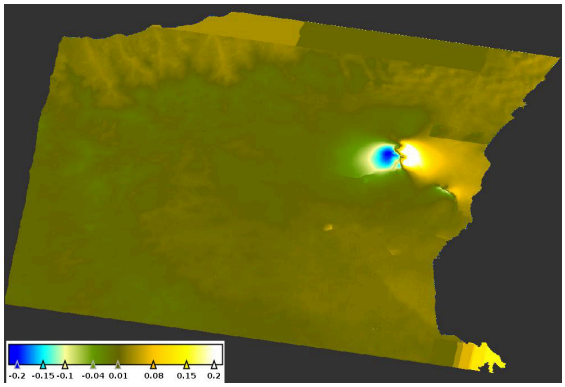


(b) Filtered interferogram (radians)

Figure 1: The original interferogram (a) and filtered interferogram (b) for the case study of the December 2018 Mount Etna eruption.



(a) Unwrapped phase (radians)



(b) Displacement estimate (metres)

Figure 2: The unwrapped phase (a) and the resulting displacement estimation (b) for the case study of the December 2018 Mount Etna eruption.

and multi-baseline phase unwrapping. The project will integrate the better performing phase denoising and unwrapping methods in the PSI pipeline, and evaluate the quality of the resulting deformation maps with respect to the standard pipeline.

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Review Article

AI and Games: The Remarkable Case of Malta

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Abstract. We currently witness a technological revolution that is attributed primarily to artificial intelligence (AI) advancements. Even before this AI Spring, however, the plethora of machine learning, search and optimization breakthroughs have been made possible through the direct applications of AI algorithms in digital games. As AI advances, games also advance since AI can continuously test, improve, design and complexify the environments it plays. This symbiotic relationship between AI and games is currently shaping the research frontier of AI and boosts the innovation potential of games across multiple domains. This short paper has a dual purpose and corresponding parts. Throughout the first *general* part of the paper, I survey briefly the current state of the art in the AI and games field. Then I outline the critical role of games in AI research, the importance of AI for game development, and the impact their relationship has on current and future scientific breakthroughs. In the second *specific* part of this paper, I focus on the Institute of Digital Games of the University of Malta as a successful centre of excellence on AI and games research, education, and innovation. Specifically, I provide evidence suggesting that a national focus and targeted investment in AI and video game development has managed to place a small island country like Malta—in just under a decade—among the leading players of AI and games research, education and innovation globally.

Keywords: Artificial Intelligence, Games, Malta

1 Introduction

Artificial Intelligence (AI) is arguably the leading driving force in what we experience as the 4^{th} industrial revolution nowadays. Since the birth of the very idea of AI, games have been the key enabler of AI breakthroughs including deep learning and artificial general intelligence (Yannakakis et al., 2018). It is not only AI that advances through games, however; AI has been assisting games to evolve in the ways we play them, test them, design them, and in the ways we understand play, learning, interaction and creativity. As games get increasingly richer and more complex through creative AI processes, AI advances further and, in turn, it advances the environments it is trained on, in a continuous and evolving relationship. The methods and technology we build *through* and *for* video games today will run the world of tomorrow: from self-driving cars, manufacturing and cyberphysical systems, to social media and even the *metaverse*. Video games are perhaps the most important domain to develop AI for, while AI is arguably the most important technological leap forward for games.

The aim of this paper is two-fold: first, it puts an emphasis on the importance of the AI and games research field as a whole and the significant impact this field has had beyond AI research and game development; second, it considers the particular case of Malta—representing a small island state and the academic residence of the author—and explores the significant scientific, research and socioeconomic effects the AI and games field has had on the country as a whole. On that basis, in the first part of the paper, I outline the advancements that games have offered to AI research, the ways AI has boosted game technology and I conclude with an observation about their continuous co-evolution which drives technological advancements across numerous domains and research fields. In the second part of the paper I focus on the impact the field of AI and games has had on the research, educational and innovation ecosystem of Malta, through the establishment of the *Institute of Digital Games*[1]. In just under a decade, AI and games have become a key enabler of research and technological innovation on a small island state that experiences high degrees of economic vulnerability (Moncada et al., 2021). Building on and benefiting from the national focus on AI and video game

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development, and the flexibility and versatility of the University of Malta—the oldest and largest small state university in the world (Moncada et al., 2021)—the Institute of Digital Games has put the University of Malta and the country on the world-map of technical games research (Nelson, 2022). Importantly, the AI and games research and educational activities performed at the Institute of Digital Games have helped the country excel in exceptional ways across a number of key socioeconomic factors and research and innovation indexes as set by Malta and the European Commission.

2 Part I: Artificial Intelligence and Games

In this first, general, part of this paper I will outline the ways AI and games have benefited from each other—games for AI versus AI for games. I will conclude this first part by emphasizing the constant co-evolution between the domain (games) and the field (AI) and the significant impact of this symbiotic relationship on future scientific discoveries and technical advancements.

2.1 Games for AI

The dominant use of AI within games and simulated environments has been for playing games well, ideally better than humans. Ever since the birth of AI, over 70 years ago, games are used to test the capacity of algorithms to perform tasks better than humans. It is a widely adopted idea that games offer challenging tasks to humans which, in turn, require some form of intelligence. Designing algorithms that are able to beat humans in games including Chess (Campbell et al., 2002), Go (Silver et al., 2017), Atari games (Mnih et al., 2015), Racing (Wurman et al., 2022) and strategy games (Berner et al., 2019; Vinyals et al., 2019) have defined the core milestones of AI research. Algorithms such as variants of tree search and Monte Carlo tree search (Checkers and Go), reinforcement learning (RL) and deep RL (Atari, Go, Dota 2, and StarCraft), multi-agent deep RL (StarCraft), and combinations of those, have been invented through research on AI gameplaying (Risi et al., 2020a). Those algorithms were able to beat the best of humans in a particular game; however, we are far from AI beating human players in every game out there.

Studying the capacity of systems that are capable of performing well across tasks is a long-standing goal of AI. In the domain of games, think of AI algorithms that are capable of playing not just a specific game, but any given game. The idea of artificial general intelligence was unsurprisingly first tested within board games (Genesereth et al., 2005; Thawonmas et al., 2019; Yannakakis et al., 2018) and has evolved nowadays to the study of generalised agents that are capable of playing different video games (Perez-Liebana et al., 2015; Reed et al., 2022; Silver et al., 2018).

It is easy to explain why games have been (and will be) so popular and important for AI research: they are well-controlled environments that can be simulated rapidly. This makes AI testing more efficient as the focus is put on the AI algorithm rather than the real world implications of AI systems. Beyond these unique properties, however, games very often represent facets of the real world. Thus certain aspects of what we learn in games, including decision making, control, optimization and behaviour, can be transferred to real-world applications. From research on computer vision (Trivedi et al., 2021; Trivedi et al., 2022) all the way to autonomous car driving (Kim et al., 2021; Martinez et al., 2017) and architecture (Radford, 2000; Xylakis et al., 2021), games can rapidly test and improve any AI approach before it is transferred into the wild.

2.2 AI for Games

What is often surprising to many AI researchers is that the AI and games field (or game AI for simplicity) is not only associated with AI-based gameplaying with the sole purpose of beating humans at a game. Thankfully there are multiple other uses of AI in games within gameplaying and beyond that are benefiting games themselves (Yannakakis et al., 2018). When it comes to gameplaying for instance, AI can be extremely useful for testing games (Holmgård et al., 2014), for imitating the ways humans behave and experience a game (Yannakakis et al., 2014b), for simulating human cooperation (Bard et al., 2020; Sfikas et al., 2021), and even for automatically detecting glitches of designed levels (Yannakakis et al., 2022).

Beyond gameplaying, AI finds applications primarily in the areas of game generative systems and player modelling. In the former, algorithmic frameworks including search (Togelius et al., 2011), evolutionary algorithms (Gravina et al., 2019), machine learning (Summerville et al., 2018), deep learning (Liu et al., 2021) and reinforcement learning (Khalifa et al., 2020; Shu et al., 2021) can drive the generation and evaluation of game content. The content generated can be anything existent in a game: visuals, narrative, levels, game rules, audio, and even gameplay patterns (Liapis et al., 2014). Such content can be generated autonomously or in a creative dialogue with a designer in a mixed-initiative fashion (Yannakakis et al., 2014a). Procedural content generation algorithms have been applied to a variety of game genres and game content types including tracks for racing games (Togelius et al., 2007), weapons (Gravina et al., 2016) and levels for first person shooters (Cachia et al., 2015),

strategy games (Togelius et al., 2010), platformers (Shu et al., 2021; Summerville et al., 2016) and 3-match tile games (Volz et al., 2020), audio for horror games (Lopes et al., 2015), and levels for arcade games (Perez-Liebana et al., 2019). Arguably the most popular task for procedural content generation throughout the decades of this field's existence is automated level design.

When it comes to player modelling, AI is tasked to capture aspects of player behaviour or experience (Yannakakis et al., 2018). The former is usually achieved by imitating human behavioural demonstrations including play traces and action sequences. The latter refers to the prediction of human experience demonstrations (such as arousal traces) based on multimodal data obtained from the player and the game including physiology (Chanel et al., 2011; Martínez et al., 2014), ad-hoc designed features (Melhart et al., 2021) but even player-agnostic representations such as the game footage pixels (Makantasis et al., 2019, 2021).

Testing, content generation, and player modelling have collectively changed the ways we design and develop games and have boosted the entire game production process. It is nowadays possible to automatically test parts of or entire games with only limited demonstrations (Barthet et al., 2022). It is also entirely possible to generate aspects of such games in a semi-autonomous fashion as, for instance, the *Candy Crush Saga* (King, 2012) level generator (Volz et al., 2020). One can also automate large parts of the quality assurance and user experience testing process by detecting aspects of player motivation, player engagement or toxicity in popular titles such as *PUBG: Battlegrounds* (Tencent Games, 2017) (Melhart et al., 2020), *Tom Clancy's the Division* (Ubisoft, 2016) (Melhart et al., 2019), and *For Honor* (Ubisoft, 2017) (Canossa et al., 2021). Needless to say, all aforementioned AI technology has reframed the design of monetization strategies followed within game production.

2.3 The AI and Games Co-Evolution and The Road Ahead

One might argue that the evolution of AI via games and the development of games via AI are independent lines of research and innovation. Recent studies, however, have showcased that AI and games are intertwined to such a degree that the domain (games) influences and advances the field (AI) and vice versa. This relationship, in turn, has multiplying effects and impacts several other domains beyond games. AI algorithms that build continuously larger, more challenging, multimodal and rich environments are setting continuously harder milestones for AI to achieve (Risi et al., 2020b). Moreover, AI that is able to capture human demonstrations of behaviour and experience is, in turn, able to automatically play and test unseen new environments in a human-like fashion (Barthet et al., 2021; Holmgård et al., 2014). Everybody wins with this competitive co-evolution of AI and games: game worlds become interesting, novel and increasingly complex and AI learns to complete downstream tasks of growing difficulty.

So where do we stand currently with this relationship and what does the road ahead look like? Representation learning appears to be key for unlocking technological breakthroughs such as foundation models (Bommasani et al., 2021). While the focus on games as testbeds (games for AI) has given us promising solutions to near-optimal play in complex real-time strategy games like *StarCraft II* (Blizzard Entertainment, 2010) (Vinyals et al., 2019) it seems that the next leap forward comes from advancements achieved within the representation of games. The dominant ways of approaching testing and player modelling via forms of self-supervised learning, for instance, are likely to become a game changer for the AI we develop for games and beyond. Recent studies suggest that unsupervised and self-supervised learning methods are able to represent the game state in an aesthetics-agnostic (Trivedi et al., 2021) and task-agnostic fashion (Trivedi et al., 2022) which we can then use in a generalised manner beyond games (Reed et al., 2022). These findings showcase that it is possible to create general representations of games that capture the very context of a game (e.g. "I now see a football game!") independently of the game's aesthetics (e.g. arcade, retro, photo-realistic, etc.) and the downstream task (e.g. playing a game or modeling a player). Such computer vision methods may equip us with powerful tools for representing the mechanics of games which can, in turn, be transferred and evaluated on real world applications (e.g. a real football game).

The long-term vision for AI is to be able to capture the dynamics of games and play in such a generalised manner that it would be able to play any given game made by human designers or AI (Yannakakis et al., 2018). As such, the recently celebrated metaverse is another unique opportunity for algorithmic advancements in AI. It is important to be reminded, however, that games are essentially the front-end of the metaverse; at least the first instance of what is envisaged as a multi-user immersive interactive world. It is not a surprise that game engine giants such as Unity and Unreal are invested to the development of such massively online multi-user worlds. The underlying game technology is already there and can be adopted from successful massively multiplayer online games like *World of Warcraft* (Blizzard Entertainment, 2005). The immersive and interfacing technologies like VR and AR, on the contrary, still define the core technical obstacles for enabling an immersive experience in such virtual spaces. The need

for intelligent entities (i.e. agents) that behave and experience their virtual worlds like humans will be ever growing. AI will also likely take, in part, the role of a metaverse designer, as it currently does in game design. Once human and AI authored worlds are generated and populated, AI will be tasked to test them and make sure that they are operational, that they satisfy the metaverse's rules and that they are fun to "live" in.

3 Part II: AI and Games at the Institute of Digital Games

By now it should be obvious that the interaction between AI and games has impacted the research world and has led to breakthroughs across multiple domains and disciplines. In this second part of the paper I will look through the local lens of Malta as an exceptional case study in this research field and outline a brief history of what AI and games have brought collectively to the country over the last decade. In particular, I will explore important research and development indicators that provide evidence for this nationwide impact. Such research intensity showcases, in turn, how a small island state (Baldacchino et al., 2018) that invested in highly multidisciplinary research and education has managed to reach and enjoy global recognition.

The Institute of Digital Games (IDG), established in 2013, is a unique multidisciplinary and multicultural research centre of the University of Malta (UM). Since its establishment such a centre—with a sole focus on the domain of games—offered an attractive proposition to game researchers. In particular, IDG combined uniquely i) an English speaking Institution in an English speaking country ii) a focus on the multidisciplinary domain of games; iii) the support of a national strategy on video game development; and iv) the lack of any other such centre in the vicinity (i.e. Southern Europe and the Mediterranean) at the time. Due to its unique proposition the IDG has managed to attract and host top researchers with multidisciplinary interests in games from Malta and other (mostly Mediterranean) countries. Among other notable achievements, the multidisciplinary group of IDG has managed to publish top-cited books in game studies (Calleja, 2011; Gualeni, 2015; Gualeni et al., 2020) and game design (Calleja, 2022) with top-tier publishers such as MIT press, and win awards for published games.

IDG contributes to and is benefited from other Faculties, Centres and Institutes at the UM by offering courses to a wide range of educational programs that use games in their curriculum including ICT, media, English, architecture, and psychology. The Institute also co-hosts joint-research projects and training seminars with a number of UM departments and corresponding disciplines including (but not limited to) AI, distributed ledger technologies, literature, philosophy, creative thinking and innovation, and linguistics and language technology. Importantly, IDG is a key stakeholder of GamingMalta, the independent non-profit foundation tasked with the remit of promoting Malta as a centre of excellence in digital game development[2]. Notably, IDG has assisted the Government of Malta to shape its research and innovation strategy around video game development and eSports; the Unity Centre of Excellence is an indicative initiative of the IDG[3].

Among other disciplines and fields and importantly for this paper, IDG hosts an active AI research group with an emphasis on applied game AI[4]. The group also offers graduate (MSc and PhD) education in game technology and game AI. Research on AI and games performed at the IDG feeds directly the graduate program in game design and game technology which, since 2017, is placed within the Top 25 Game Design programs worldwide by *The Princeton Review* (The Princeton Review, 2022).

During the last decade, the IDG AI group has been successful in implementing a strong research program on game AI, procedural content generation and player modeling which is demonstrated through the number of highly cited articles in globally recognized journals and conference proceedings. In particular, more than 250 papers have been published in the broader area of AI and games and have received more than 22,000 citations (h-index: 170) collectively (Institute of Digital Games, University of Malta, 2020). As a result the research outreach of IDG has reached a world-class standard currently (2022) ranked 6^{th} in the list of the top 100 technical games research institutions globally (Nelson, 2022). Beyond its rich publication output, the Institute has coordinated and managed numerous national, FP7, H2020, and Horizon Europe projects attracting a total budget of over 6m Euro for supporting its research activities in game AI and game design. According to the Malta Council for Science and Technology, the Institute also hosts the most active researcher in the country[5]. The research and innovation (R&I) projects administered by the AI group of IDG have led to outcomes that collectively have advanced AI methods for affective computing and generative systems, but also fostered the creative use of AI for media, cultural heritage, architecture and engineering. Moreover, the successful implementation of the aforementioned projects has enhanced and empowered the ways we teach, bring-

[2] https://www.gamingmalta.org/

[3] https://timesofmalta.com/articles/view/the-gamingmalta-foundation-announces-partnership-with-unity.931927

[4] https://www.um.edu.mt/digitalgames/airesearchgroup/

[5] https://www.um.edu.mt/newspoint/news/2021/02/most-active-researcher

ing innovative game technology, AI and machine learning to the epicentre of education. The projects collectively contributed to a gradual shift from digital to AI literacy and engaged students in Malta and abroad to become responsible citizens with regard to creative thinking, societal challenges and even ethical implications of AI.

Beyond its research and educational agenda, the AI group of IDG also excels in technical innovation. Its spin-out, modl.ai[6] is currently listed within the top 100 most innovative AI start-ups worldwide and the only one within the games sector (CB Insights, 2022). Several faculty and alumni of IDG are nowadays actively contributing to the company's vision to build an AI engine that would be able to test any given game rapidly via sophisticated testing technology. Indicative of its success—at the time of the writing this paper—the IDG spin-out received an investment of $8.4m led by top-tier commercial and strategic actors including Microsoft's M12 Venture Fund[7].

The impact and significance of the Institute of Digital Games for UM and the country is reflected through key research and development indicators identified by the European Commission through its European Innovation Scoreboard 2021 (European Commission, 2021). Indicatively in 2021, the IDG contributed a 6.8% of Malta's new doctorate graduates in STEM, a 69% of new foreign doctorate students and, on average, three times more doctorate students per academic (i.e. 1.67) compared to the UM average (i.e. 0.57)[8]. Importantly, in the period between 2014 and 2020, IDG managed to attract research funds of 2.1 million Euro out of the 22.4 million Euro that was collectively attracted in Malta. This contribution amounts to 9.3% of the country's research funding during the Horizon 2020 research and development framework[9]. All the above indicators are key for the R&I growth and sustainability of Malta and offer clear evidence for the leading role of the Institute and its AI research group towards improving the *research intensity* of the country as a whole.

The aforementioned research, educational and innovation outcomes have been both timely and of national importance for Malta as both AI and video games define core strategic areas of growth and development. Importantly, AI and games support all thematic areas of Malta's R&I Smart Specialization Strategy (Malta Council for Science and Technology, 2020) and core niche areas including AI, IoT, big and open data, and human-centric applications. In summary, the analysis above indicates that a targeted investment in a niche multidisciplinary research area, even at a small scale, can have a tremendous socioeconomic impact on a small island state like Malta (Baldacchino et al., 2018). Evidently, a multidisciplinary research and educational group with a key focus on AI has managed to put Malta on the world map of technical games research and innovation.

4 Parting Words

As highlighted throughout this paper, the various uses of AI for and in games have led to numerous algorithmic breakthroughs including tree search, computer vision, (deep) reinforcement learning, language models and self-supervised learning. At the same time alternative applications of AI beyond mere gameplay optimization have resulted in advancements in generative systems of game worlds, automatic testing tools of various sorts and player behaviour and experience detection systems. In recent years, we have witnessed a research symbiosis of AI and games as algorithms generate continuously more complex and interesting worlds for AI to play, test and experience. The impact of this unique relationship is already shaping the ways we educate our children, the ways we develop game-based digital twins, the buildings we design and construct, all the way to the metaverse we envision.

While in the first part of the paper I focused on the *general* impact and significance of AI and games, in the second part I explored the nationwide impact of the field in the *specific* (and rather exceptional) case of Malta. In particular, I provided evidence showcasing how a national focus on AI and games has benefited the small island state (Baldacchino et al., 2018) within less than a decade since the establishment of the Institute of Digital Games in 2013. The socioeconomic impact of AI and games research on the country is evident through several factors including key objective academic performance indicators such as citations and awards, recognized global rankings of research and educational excellence, and key factors considered by the Commission's *European Innovation Scoreboard* (European Commission, 2021) and *Malta's Research and Innovation Smart Specialisation Strategy* (Malta Council for Science and Technology, 2020). Evidently, the Institute of Digital Games is a successful paradigm for Malta directly supporting its research, education and innovation ecosystem on video games and artificial intelligence. Given its bright present, the future of games and AI research in Malta brings increased responsibility for an ever-growing research and innovation intensity with a global impact.

I wish to conclude this paper with a critical note regarding any potential future challenges the Institute of

[6] https://modl.ai/

[7] https://venturebeat.com/games/modl-ai-seriesa-ai-bot-qa-testing-griffin-gaming-microsoft-m12/

[8] Source: Doctoral School, University of Malta; https://www.um.edu.mt/doctoralschool

[9] Source: Malta Council for Science and Technology; https://mcst.gov.mt/

Digital Games might face. One might question the sustainability of the IDG given its small size and the critical impact most members of staff have on its success. Arguably it is a weakness for a small Institute—being part of a country's single University—to be dependent on a few successful academics. One would argue, however, that its size is also one of its core strengths when it comes to resource management and daily operations. Given the Institute's growing reputation in games research it should be emphasized that attracting world-class researchers to Malta—for further supporting IDG's activities—should be considered guaranteed by now. The long-term availability of external funding across many disciplines beyond AI (e.g. digital humanities, education, cultural heritage, extended reality) improves and strengthens the position of IDG as a self-sustained unit. Despite being small and hosted in a small island country, IDG will likely continue to thrive as supported by its established reputation, and its versatile and multifaceted (i.e. research, education, innovation) impact across diverse disciplines within games.

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Review Article

Precision Medicine and Enrichment in Sepsis

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Abstract. Sepsis is defined as a dysregulated host response to infection leading to life-threatening organ dysfunction. While this recent iteration of the sepsis definition rightly centralizes organ dysfunction, it does not reflect on the extensive heterogeneity in the host response observed in sepsis patient populations. Heterogeneity in sepsis has hindered the identification of effective therapeutic targets, with current treatment consisting of antimicrobials and supportive care. In order to address the shortcomings in identifying specific therapeutics for sepsis, the focus of various research activities turned towards developing precision medicine approaches. In particular, efforts aimed at stratifying patients into more homogenous subgroups having common dominant pathophysiological features and outcome trajectories, in turn facilitating the delineation of specific therapies. Here, I review current initiatives in prognostic and predictive enrichment strategies in sepsis patient populations, which will be key to identify patients who would benefit, or be harmed, from specific therapeutic interventions.

Keywords: Sepsis, Stratification, Precision, Treatment, Intensive care

1 Introduction

The word "sepsis" was derived from the ancient Greek word "sepo" (σήπω), meaning "I rot". Around 400 BC, Hippocrates described sepsis as the process of hazardous biological decay that could happen in the human body. In the 19th century, with the discovery of microorganisms as causal agents of infection, sepsis was described as a condition associated with severe infections. Since then, the terminology and definitions of sepsis have gone through various iterations. It is a clinical syndrome, not a disease (Vincent et al., 2013), currently defined by consensus as "life-threatening organ dysfunction caused by a dysregulated host response to infection" (Singer et al., 2016). A severe complication of sepsis, that is septic shock, is characterized by vascular hypotension, critical tissue perfusion aberrations and major organ failure, with in-hospital mortality rates reaching as high as 50% (Angus et al., 2013; Hotchkiss et al., 2016). There is no archetypal sepsis patient as it hits individuals indiscriminately across age groups, genders, races, ethnicities or geographical locations. Respiratory or intra-abdominal infections are major determinants of sepsis in elderly and neonatal populations (Rudd et al., 2020). In 2017, sepsis accounted for an estimated 11 million deaths worldwide, equating to age-standardized mortality rates of 148 per 100,000 population (Rudd et al., 2020). The incidence of all-cause sepsis in Malta was estimated at 1349 individuals, responsible for approximately 272 deaths in 2017 (Rudd et al., 2020). While age-standardized incidence and mortality have declined between 1990 and 2017 (Rudd et al., 2020), mainly attributable to advances in antimicrobial therapy and supportive care (Prescott et al., 2018), the incidence of sepsis remained stable and survivors continue to suffer from additional morbidities and poor outcomes (Iwashyna et al., 2010; Shankar-Hari et al., 2016). About 50% of patients who survive sepsis are re-admitted to hospital at least once within the first year, and approximately one-third die (Prescott et al., 2018). The alarming incidence and mortality rates prompted the World Health Organization to adopt a resolution recognizing sepsis as a global health priority (Reinhart et al., 2017). It is expected that sepsis will remain a global problem due to a combination of factors, including a progressively ageing population, surgical interventions, potent immunosuppressive drugs, antimicrobial resistance and emergence of viruses with pandemic potential. Despite remarkable advances in our understanding of the sepsis pathophysiology, par-

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ticularly immunopathological aspects (van der Poll et al., 2017), no specific drug that substantially mitigates poor outcomes has been approved. Numerous clinical trials targeting components of the host response were unsuccessful (Marshall, 2014). Those shortcomings have been ascribed to extensive heterogeneity of the sepsis syndrome, which hinders the identification of patients who would benefit, or be harmed, by specific therapeutic adjuvants, thus motivating current attempts to establish a precision medicine strategy to sepsis diagnosis and treatment. Throughout the last two decades, considerable progress has been made in the stratification of sepsis patients as subgroups by means of host response parameters. Such strategies have been proposed as potentially critical tools to improve on therapies that target specific pathophysiological mechanisms (Marshall, 2014; Stanski et al., 2020). Here, I outline the efforts that have been made to resolve the heterogeneity of sepsis, using either clinical parameters or genomics data, or combinations thereof.

2 Precision Medicine

The term precision medicine refers to the concept that fundamentally moves diagnosis, prognosis and treatment strategies away from the "one-size-fits-all" mindset, that is taking into consideration individual patient characteristics (Collins et al., 2015; Wong, 2017). This personalized approach was conceptualized in the field of oncology, which has advanced greatly in comparison to sepsis. Technological innovations in genomics, transcriptomics, proteomics, epigenomics and immune profiling have enabled the identification of tumour molecular markers that can be targeted with tailored therapeutic agents. For example, the Initiative for Molecular Profiling and Advanced Cancer Therapy (IMPACT) studies (Tsimberidou et al., 2012; Tsimberidou et al., 2014). Targeted molecular therapies that include immune checkpoint blockade inhibitors and anti-programmed cell death (PD)-1 have anti-tumour activity against numerous types, including melanoma, lung, breast and bladder cancers, however only 10%–30% of patients benefit from these immunotherapeutic agents (Larkin et al., 2015). Hence, development of molecular biomarkers to identify those patients who would benefit from tailored treatment strategies are key to establishing precision medicine approaches (National Academies Press (US), 2016). In recognition of important advances in cancer treatment facilitated by making use of precision medicine methods, the federal government of the United States launched the "Precision Medicine Initiative" in 2015 (White House, 2015). The Malta National Cancer Plan, launched by the Ministry for Health in 2021, also recognized the importance of developing precision medicine to aid in cancer treatment strategies (Ministry of Health, 2021). Against this backdrop, the natural next step would be the application of precision medicine approaches to other heterogeneous diseases and syndromes, such as sepsis. To this end, a currently ongoing proof-of-concept "Personalized Immunotherapy in Sepsis: a Multicentre and Multinational, Double-blind, Double-dummy Randomized Clinical Trial" (IMMUNOSEP; ClinicalTrials.gov identifier: NCT04990232), coordinated by the Hellenic Institute for the Study of Sepsis, seeks to provide benchmark evidence for the application of precision medicine principles in the field of critical illness due to sepsis. The concept of "enrichment" is a key principle of precision medicine (Wong, 2017), which refers to reducing population level heterogeneity. It is subcategorized as either prognostic or predictive enrichment of patient populations (figure 1). Prognostic enrichment describes selection of a subgroup of patients who are more likely to meet relevant outcomes or clinical endpoints, for example mortality (Prescott et al., 2016; Stanski et al., 2020). An example of prognostic enrichment is the successful "Effect of Prone Positioning on Mortality in Patients With Severe and Persistent Acute Respiratory Distress Syndrome (ARDS)" trial (PROSEVA; ClinicalTrials.gov Identifier: NCT00527813) (Guérin et al., 2013). The PROSEVA trial of proning therapy enrolled only those patients with an arterial to inspired oxygen ratio (PaO_2/FiO_2) less than 150 mmHg, hence enriching the trial for patients with moderate-to-severe ARDS. The trial showed that prolonged prone-positioning significantly increased survival in patients with severe ARDS (Guérin et al., 2013). Predictive enrichment refers to the selection of a subgroup of patients who are more likely to respond favorably to a given treatment targeting a specific biological mechanism relative to unselected patients (Prescott et al., 2016; Stanski et al., 2020). A typical example of predictive enrichment is shown in the successful use of trastuzumab, a recombinant monoclonal antibody targeting human epidermal growth factor receptor 2 (HER2), in patients with HER2-positive breast cancer in the Herceptin Adjuvant (HERA) trial (Piccart-Gebhart et al., 2005). In the context of sepsis, predictive enrichment is challenging due to relatively limited knowledge of the dominant pathobiological mechanisms driving sepsis. In general, there is consensus among researchers and clinicians that to successfully establish precision medicine in sepsis necessitates simultaneous prognostic and predictive enrichment (Shankar-Hari et al., 2019; Stanski et al., 2020). In order to move towards this goal, it is crucial to gain a deeper understanding of the pathobiological mechanisms underlying the sepsis syndrome, which will not be achieved by considering the typical translational research model using inappropriate animal models and patient se-

lection criteria (Cavaillon et al., 2020), but by developing interdisciplinary strategies to disentangle drivers of heterogeneity in sepsis, and in turn utilizing the information to stratify patients into robust treatable subgroups.

3 Patient Stratification in Sepsis

Recent efforts to develop precision medicine strategies for sepsis have leveraged on the concepts of unsupervised clustering and machine learning to stratify sepsis patients as subgroups using several demographic, clinical and/or molecular parameters. Here, the terminology tends to be inconsistent. For clarity, patient subgroups identified by using routine clinical measurements, not necessarily reflecting a potential underlying biological mechanism, are defined as "subphenotypes", whereas "endotypes" indicate biological subtypes defined by distinct pathophysiological mechanisms. Several attempts have been made in recent years to split sepsis patient populations into subgroups, using clinical parameters and/or molecular measurements as data inputs in machine learning approaches, as well as the more traditional unsupervised clustering techniques, for example k-means or latent class analysis (DeMerle et al., 2021; Reddy et al., 2020). These initial attempts were pioneered by Hector Wong and colleagues in pediatric sepsis (Wong, 2022), who have provided benchmark evidence that prognostic and predictive enrichment strategies can unlock precision medicine in sepsis and septic shock.

3.1 Prognostic enrichment

A classic example of prognostic enrichment in sepsis is the Pediatric Sepsis Biomarker Risk Model (PERSEVERE) (Wong et al., 2012). On the basis of a discovery approach utilizing genome-wide gene expression profiling of blood leukocytes purified from pediatric septic shock patients, Wong and colleagues derived a candidate panel of 12 serum protein biomarkers for patient stratification and outcome prediction. Protein biomarkers were measured in serum samples from a cohort of 220 unselected children presenting with septic shock. Serum samples were obtained during the first 24 hours of admission to the intensive care unit (ICU). Applying Classification and Regression Tree (CART) analysis on both serum biomarkers and routinely available clinical variables a decision tree was built to predict 28-day all-cause mortality. This approach also reduced the dimensionality of serum biomarkers to a panel of 5 proteins, namely C–C chemokine ligand 3 (CCL3), Interleukin (IL)–8, granzyme B, heat shock protein (HSP) 70 kDa member 1B and matrix metallopeptidase (MMP)–8 (Wong et al., 2012). In the derivation cohort, sensitivity, specificity, negative and positive predictive values equated to 91% (95% confidence interval (CI): 70–98), 86% (95% CI: 80–90), 43% (95% CI: 29–58), and 99% (95% CI: 95–100), respectively (Wong et al., 2012). In the test (validation) cohort, sensitivity and specificity equated to 89% (95% CI: 64–98) and 64% (95% CI: 55–73), respectively. This model has been prospectively validated in other cohorts (Wong et al., 2014b), including adult septic shock patients (Wong et al., 2014a). The model progressed through additional reiterations that include thrombocyte counts to the original candidate biomarker panel (PERSEVERE-II) (Wong et al., 2016), as well as having leukocyte gene expression data included in the model (PERSEVERE-XP) (Wong, 2017). These studies provide compelling examples of the clinical utility of decision-making models built on combinations of clinical and molecular data. Another example of a combinatorial strategy in prognostic enrichment is a study that combined demographic (patient age), clinical (hematocrit, serum lactate measurements) and circulating metabolite levels, namely 4-cis-decenoylcarnitine, 2-methylbutyroylcarnitine, butyroylcarnitine and hexanoylcarnitine, obtained from patients on hospitalization for the development of a model to predict survival from sepsis (Langley et al., 2013). A support vector machine (SVM) was utilized to develop a weighted prediction model of sepsis survival, which resulted in a receiver-operator-characteristic (ROC) area-under-the-curve (AUC) of 0.819 and 0.74 in the training and validation cohort, respectively. In a community approach, a group of researchers assembled various patient cohorts with genome-wide gene expression data from whole blood leukocytes and clinical parameters, including Acute Physiology and Chronic Health Evaluation (APACHE) and Sequential Organ Failure Assessment (SOFA) scores, to the aim of identifying a gene set that is able to predict mortality due to sepsis (Sweeney et al., 2018b). The group trained four models using 12 cohorts as discovery set encompassing 485 survivors and 157 non-survivors. Model performance was tested in 9 heterogeneous validation cohorts that included 419 survivors and 52 non-survivors. Using ROC-AUCs as metrics of model performance, a joint model that included gene expression profiles and clinical indices of severity performed better than gene expression indices in isolation; albeit with extensive variability in ROC AUCs ranging from 0.537 to 1.0 in the discovery cohorts (Sweeney et al., 2018b). Other investigators combined host genetics, systemic metabolite levels and cytokine measurements in patients, to the goal of identifying a mortality predictor with pathophysiological implications (Wang et al., 2017). By also testing their findings in a mouse model, Wang and colleagues uncovered a role for the methionine salvage pathway in the pathophysiology of sepsis. High plasma concentrations

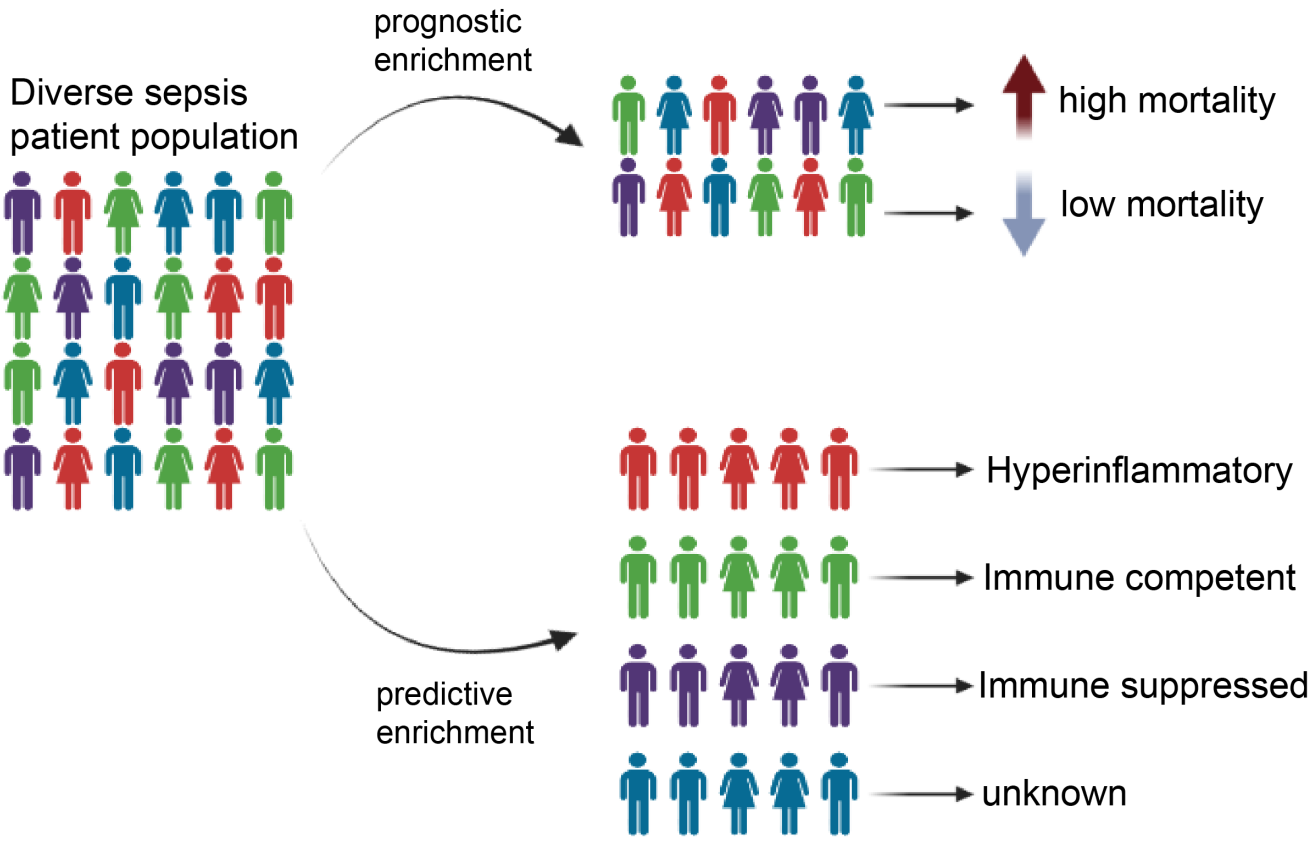

Figure 1: Illustration of prognostic and predictive enrichment of sepsis patient populations. A diverse population of critically ill patients with sepsis is analyzed in two ways: (1) prognostic enrichment to identify a subphenotype of patients at risk of adverse clinical endpoints or outcome, for example mortality, and (2) predictive enrichment to cluster patients as endotypes reflecting dominant pathobiologies amenable to specific therapeutic interventions, for example immune compromised patients treated with immune activating therapies. Both approaches to enrichment are not intended to run in isolation, but rather in combination to identify risk subgroups with underlying biological mechanisms.

of methylthioadenosine were associated with mortality in patients with sepsis and was significantly correlated with pro-inflammatory cytokine concentrations. By combining plasm levels of methylthioadenosine and other variables the investigators showed their combinatorial method could predict death due to sepsis with 80% accuracy (Wang et al., 2017). Researchers have also used readily available clinical and routine laboratory test results for prognostic enrichment. In a retrospective analysis, Seymour and colleagues identified four subphenotypes (designated α, β, ɣor δ) by utilizing data obtained from 16,552 unique patients who met Sepsis-3.0 criteria (Singer et al., 2016), within 6 hours of presentation at the emergency department in twelve Pennsylvania, USA, hospitals utilizing 29 variables in a k-means consensus clustering approach (Seymour et al., 2019). With a prevalence of 33%, the αsubphenotype was the most common and included patients with the lowest organ dysfunction and 2% mortality rate. The βsubphenotype (prevalence = 27%) had older patients with more chronic illness and renal dysfunction, as well as 5% mortality rate. The ɣsubphenotype (prevalence = 27%) included patients exhibiting hyperinflammatory patterns, higher core temperatures, more pulmonary dysfunction and 13% mortality rate. With a prevalence of 13%, the δsubphenotype was the most severe with patients presenting higher serum lactate levels, more hypotension and a mortality of 32%. When investigators considered all cohorts and trials, both 28-day and 1-year mortality rates were significantly highest among patients classified as δsubphenotype, relative to the other 3 subphenotypes (Seymour et al., 2019). Notably, Monte-Carlo simulations showed that the proportion of randomized control trials (RCTs) reporting benefit, harm, or no effect changed substantially by taking into account varying frequencies of the four subphenotypes in the study population, suggesting that α, β, ɣor δsubphenotypes may aid in better understanding the heterogeneity of treatment effects (Seymour et al., 2019). A Monte-Carlo simulation is a computational method that is used to understand the risk of a particular outcome given the presence of random variables that introduce uncertainty (Harrison, 2010). In practice, the model generates numerous results by assigning multiple values to an uncertain variable, for example treatment benefit or harm, which are subsequently averaged to obtain an estimate. Other investigators examined core temperature trajectories of sepsis patients, delineating four subphenotypes with distinct mortality risks, termed "hyperthermic, slow resolvers", "hyperthermic, fast resolvers", "normothermic" and "hypothermic" patient subphenotypes (Bhavani et al., 2019). The "hypothermic" subphenotype had the highest mortality risk concomitant with the lowest levels of inflammatory markers. Although the subphenotypes delineated by clinical parameters alone do not reflect dominant biological mechanisms, they may provide a more practical and feasible approach to risk stratification of patients with sepsis since routinely acquired clinical variables do not require advanced molecular techniques or sophisticated data analysis strategies.

3.2 Predictive enrichment

In contrast to prognostic enrichment, predictive enrichment does not utilize demographic, clinical parameters, indices of severity, and/or outcome to stratify patients into subgroups. Predictive enrichment seeks to stratify patients into biologically meaningful subgroups or "endotypes", based on unbiased computational approaches that leverage on molecular patterns representing underlying biological mechanisms. The ultimate goal of this approach is to define biomarkers that inform the attending physician on a key pathobiological feature that is potentially amenable to therapeutic intervention, that is a treatable trait (Russell et al., 2017; Scicluna et al., 2019). Genome-wide whole blood leukocyte gene expression studies are the most common strategies in predictive enrichment. So far, four clustering methods have been used in different clinical contexts to classify patients with sepsis based on whole blood leukocyte gene expression patterns, including pediatric septic shock, adult sepsis secondary to pneumonia and all-cause adult sepsis, outlined below. Hector Wong and colleagues were the first to report subgrouping of pediatric septic shock cases by means of leukocyte gene expression profiling (Wong et al., 2009; Wong et al., 2011). Patients were initially classified as either subclass (endotype) A, B or C on the basis of k-means clustering of 6,934 genes. Using a leave-one-out cross-validation strategy, a 100-gene set was derived having the highest predictive value for the delineation of the three endotypes. Evaluation of the association between endotype assignment and clinical parameters revealed patients classified as endotype A were younger, more severely sick and had higher mortality rates relative to patients classified as endotypes B or C (Wong et al., 2009). Biological pathway analysis revealed endotype A was characterized by reduced expression of genes involved in adaptive (lymphocyte) immunity and glucocorticoid receptor signaling. Notably, and in line with pathway analysis, treatment of patients assigned to endotype A with corticosteroids was associated with higher risk of mortality (Wong et al., 2015). Thus, this predictive enrichment strategy demonstrates detrimental effects of corticosteroid treatment in a proportion of septic shock patients, which lends further weight to the controversy surrounding corticosteroids being prescribed without

consideration of the underlying immune status of the patient. Moreover, this study was the first report on the potential for transcriptomic endotypes as treatable traits. Investigators from the United Kingdom enrolled adult patients with sepsis secondary to community-acquired pneumonia in the Genomic Advances in Sepsis (GAinS) study, and analyzed leukocyte gene expression data by unsupervised hierarchical clustering of the top 10% most variable genes (n=2619 genes). In doing so, two endotypes or sepsis response signatures (SRS) 1 and 2 were identified in a discovery cohort of 256 patients (Davenport et al., 2016). Patients assigned to SRS1 (prevalence = 41%) were more severely ill and at higher risk of mortality as compared to SRS2 patients (prevalence = 59%). Biological pathway analysis revealed SRS1 was characterized by genes involved in endotoxin tolerance, T cell exhaustion and reduced expression of genes linked to the major histocompatibility complex class II (Davenport et al., 2016). In a follow-up study that included patients diagnosed with sepsis due to fecal peritonitis, SRS 1 and 2 signatures were validated (Burnham et al., 2017). A seven gene signature was derived for the classification of patients as either SRS1 or 2, namely DYRK2, CCNB1IP1, TDRD9, ZAP70, ARL14EP, MDC1, and ADGRE3 (Davenport et al., 2016). Moreover, the same research group performed a post hoc analysis of a double-blind, randomized clinical trial in septic shock, that is the Vasopressin vs. Norepinephrine as Initial Therapy in Septic Shock (VANISH; Clinicaltrials.gov identifier: ISRCTN 20769191) trial. The group reported no association with vasopressor choice, however, corticosteroid treatment prescribed to patients classified as the relatively low-risk, immunocompetent endotype SRS2 was associated with increased mortality and an adjusted odds ratio of 7.9 (95% CI: 1.6–39.9) (Antcliffe et al., 2019). Therefore, the proposed interaction between steroid treatment and the relatively less-severe SRS2 endotype implies opposing effects of the same therapeutic intervention across and also within distinct patient endotypes (Scicluna et al., 2019). Replication of these findings is certainly needed, particularly because a recent re-analysis of the VANISH trial showed that steroid treatment was associated with increased mortality in adult septic shock patients classified as (pediatric) endotype A, which was shown to have similarities with immune compromised SRS1, not immune competent SRS2 (Wong et al., 2021). In the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study, other investigators analyzed whole blood transcriptomic data (n=5000 genes) obtained from adult patients with all-cause sepsis on ICU admission, that is sepsis due to different infectious etiologies, identifying four molecular endotypes termed MARS1 to MARS4 (Scicluna et al., 2017). The MARS1 endotype was associated with poor prognosis, having high total SOFA scores, higher prevalence of septic shock (44%), and mortality rates reaching 39% after 28-day patient follow-up. The MARS3 endotype was relatively less severe, with patients having lower SOFA scores and septic shock presentation (17%), with 28-day mortality rate equating to 23% (Scicluna et al., 2017). By combining APACHE IV scores and molecular endotype assignment in an analysis of the net reclassification improvement showed this clinicomolecular model significantly improved risk prediction relative to only considering clinical risk prediction. Biological pathway analysis revealed the poor prognosis MARS1 endotype was associated with reduced innate and adaptive immune functions attuned to an immunosuppressed state, whereas gene expression profiles of the low-risk MARS3 endotype were consistent with elevated capacities for adaptive immune reactions, particularly increased lymphocyte functions (Scicluna et al., 2017). The capacity to identify immunosuppressed patients is especially appealing since these patients will not benefit from steroid treatment, but more likely to respond favorably to therapeutic interventions aimed at restoring immune function, for example Interferon-γ treatment, which has been shown to reverse immune paralysis in a small cohort of sepsis patients (Cheng et al., 2016). The MARS investigators proceeded to derive a 140-gene classifier that enabled the validation of the MARS endotypes in two additional cohorts, including the previously described GAinS cohort (Davenport et al., 2016). To facilitate translation to the clinic, MARS investigators refined their gene expression classifier to a panel of eight genes (BPGM, TAP2, GADD45A, PCGF5, AHNAK, PDCD10, IFIT5 and GLTSCR2). Comparing MARS and SRS endotype membership demonstrated significant overlap between the low-risk endotypes MARS3 and SRS2 (Davenport et al., 2016; Scicluna et al., 2017). Moreover, MARS investigators also tested their endotype classification strategy in the aforementioned pediatric septic shock cohort (Wong et al., 2009). The relatively low-risk MARS3 endotype, characterized by gene expression patterns attuned to heightened adaptive immune responses, was not reliably delineated in the pediatric sepsis cases. The investigators argued that the selection of septic shock patients in the pediatric cohort, as well as an under-developed adaptive immune system in children may explain the lack of MARS3 assignments (Scicluna et al., 2017). The complicated relationship between patient age and endotype classification is notable, which was demonstrated in a study that sought to classify adult sepsis patients to pediatric endotypes (Wong et al., 2017). These observations suggest that a unifying model across patient ages may not be feasible, but dis-

tinct classification strategies for pediatric and adult patients may be necessary. Pooling gene expression data from 14 bacterial sepsis cohorts (n=700), including pediatric and adult patients admitted to hospitals in seven countries, Sweeney and colleagues used two clustering algorithms, that is k-means clustering and Partitioning Around Medoids (PAM), identifying three transcriptomic endotypes (Sweeney et al., 2018b). The three endotypes were termed "inflammopathic", "adaptive", and "coagulopathic". Considering results of both discovery and validation sets, the "adaptive" endotype was associated with a lower clinical severity and lower mortality rate. In contrast, the "coagulopathic" endotype was associated with older age, higher mortality and coagulation dysfunction (Sweeney et al., 2018a). Similarities between classification strategies was also reported, specifically the "inflammopathic" endotype overlapped SRS1 and pediatric endotype B. The "adaptive" endotype corresponded to the SRS2 endotype. On the basis of a 33-gene classifier to assign each endotype ("inflammopathic", "adaptive", or "coagulopathic"), 97 patients with coronavirus infectious disease (COVID) 2019 (Sweeney, Timothy E. et al., 2021), the disease caused by Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2). COVID-19 patients were assigned to "inflammopathic" (29%), "adaptive" (44%), or "coagulopathic" (27%) endotypes, with similar proportions to the previous study in bacterial sepsis (Sweeney et al., 2018a). Notably, patients assigned to the "adaptive" endotype were less severe and no deaths, whereas "coagulopathic" and "inflammopathic" endotypes were more severe, having mortality rates equating to 42% and 18%, respectively.

4 Future perspectives

The application of prognostic and/or predictive enrichment strategies to sepsis patients have the potential to provide much-needed precision to diagnosis, treatment and clinical trial design. Much work is needed for the field to progress to the same extent as in oncology. An important step towards the ultimate goal of predictive and prognostic enrichment, that is risk stratification of sepsis patients into subphenotypes or endotypes for use in the clinic, will require international collaborative efforts to establish a consensus sepsis endotype model. The importance of a consensus model is exemplified by work in the field of colorectal cancer. By combining high-dimensional gene expression studies from six independent research groups, all with their own subtype classification strategies, a consensus four-subtype model was developed that standardized colorectal cancer subclassification for further studies (Guinney et al., 2015; Linnekamp et al., 2018). While the observed similarities between the earlier-mentioned methods for sepsis patient stratification as transcriptomic endotypes are reassuring, we do not know whether overlapping endotypes describe the same subgroup of patients. It is essential to investigate the similarities and differences between patient endotypes, particularly across different geographical populations. To date, most studies were limited to people of Northern European ancestry, which certainly restricts our ability to identify sources of inter-individual variation in the host response, and consequently generalizability of patient endotypes. Thus, including more diverse patient populations in stratification studies will go a long way to developing veritable consensus sepsis endotypes. Until now, the vast majority of genomics studies for the purpose of patient stratification utilized whole blood leukocyte transcriptomes obtained on ICU admission. While whole blood is extremely relevant biological specimen to the clinic owing to its accessibility, it does complicate the interpretation of transcriptomic endotypes. Whether the leukocyte gene expression patterns observed in sepsis patients on ICU admission reflect dominant pathobiological mechanisms, especially those that ensue at the primary anatomical site of infection is unknown. Establishing a connection between organ-specific pathophysiology in sepsis and transcriptomic endotype membership cannot be overstated. It is crucial to better understand the relationship between transcriptomic endotypes, particularly those that emerge from consensus endotype efforts, and organ-specific biology in sepsis. In addition, it is envisaged that future studies will be designed in a longitudinal manner, that is obtaining specimens at various time points of a patient's ICU stay. The host response to infection is a highly dynamic and temporally coordinated process, exemplified by the time-dependent patterns observed in the human endotoxemia model (Perlee et al., 2018; Scicluna et al., 2020). This is particularly pertinent to sepsis endotypes studies since it was observed that approximately 30% of children with septic shock switch endotype membership during the first 72 hours after ICU admission (Wong et al., 2018).

5 Concluding Remarks

Technological advances have heralded important discoveries in sepsis pathophysiology, particularly in the immunology of sepsis (van der Poll et al., 2021). Despite an improved understanding of the immunopathology of sepsis, translation to effective treatments remains problematic. It is evident that unraveling the complexities that underlie the heterogeneity in sepsis is a challenging task, requiring more than reductionist approaches alone. Embracing the concepts of integrative biology, that is bringing together investigators of diverse specialties, for example anatomy, physiology, biochemistry, pathology, mo-

lecular biology, genetics, genomics and mathematics to address the problem of sepsis not only in a multidisciplinary manner but also transdisciplinary. The current method of choice is utilizing high-dimensional "omics" data and data science for immune-profiling in a "multi-omics" approach, permitting analysis of multiple molecular strata that include DNA, RNA, proteins and metabolites from the same sample. While useful in building platforms for the derivation of new hypotheses, it will be critical to build these models not only using systemic molecular profiles, but also at the tissue-site of infection (Cavaillon et al., 2020). More attention should be given to designing longitudinal "multi-omics" studies, including samples not only obtained during a patient's ICU stay, but also after hospital discharge. This approach will allow for a more holistic integrative model of the septic response, patient trajectories and the long-term consequences. In this way, host response biomarkers will be derived reflecting dominant pathobiological mechanisms during the acute and/or convalescent phases, which despite its challenges is envisaged to progress to realizing the promise of precision medicine approaches in sepsis.

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Research Article

Impacts of local and landscape habitat variables and honeybee visitation on wild bee diversity in the Maltese Islands

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Abstract. Bees are important pollinators in several ecosystems, and losses of bee diversity can impact on crop and wild plants pollination, and associated ecosystem services. Here, we explore how landscape and local habitat variables influence honeybee and wild bee functional group abundance and richness. Within the context of high hive densities in the Maltese Islands, we explore how high honeybee visitation impacts on wild bee functional richness and abundance using a dataset of plant-bee networks from 78 sites surveyed in spring 2019. Honeybees were strongly associated with agricultural habitats and used a wide range of floral resources that overlapped with those used by wild bees. While no significant impact of local habitat type on functional group richness was recorded, different functional groups were associated with different local habitat types, with agricultural, garden and roadside vegetation habitats being the most important habitats. We assessed the influence of landscape context on wild bees in two radii (250 and 500 m). At these scales, the land cover that influenced wild bee abundance positively were arable, garrigue and grassland, orchard and urban, and the abundance of different bee groups was influenced differently by landscape parameters. High honeybee visitation rate had a negative impact on wild bee abundance but no significant impact on functional group richness was recorded in this study. These results are used to provide recommendations for habitat management for bee conservation, as we stress the need for a more holistic approach that considers the effect of local and landscape habitat characteristics, and interspecific interactions when planning measures for the conservation of bee diversity and pollination ecosystem services in the Maltese Islands.

Keywords: Apoidea, ecosystem services, floral resources, honeybee abundance, pollination, plant-bee networks

1 Introduction

Biotic pollination is an important regulating service, upon which 87.5% of the world's flowering wild plants, and 85% of the leading crops depend, at least in part, while pollinators also contribute to multiple ecosystem services that lead to ecological, cultural, financial, health, human, and social benefits. Globally, pollination boosts yields and quality of cultivated crops by an estimated US$235-577 billion to global food production value (IPBES, 2016). Moreover, pollinators provide a wide diversity of benefits that goes beyond food production, including biofuels (e.g., canola and palm oils), fibres (e.g., cotton), forage for livestock, fibres, health (e.g., pharmaceutical properties of bee products), materials for candles, musical instruments, arts, and crafts, and other benefits to communities (e.g., employment and beekeeping) (Potts et al., 2016). Declines in pollinating insects have been reported globally for a range of taxonomic groups (Sánchez-Bayo et al., 2019), and have also been recorded in the plants on which they rely (Biesmeijer et al., 2006; Cardoso et al., 2020), raising concerns of a global pollinator crisis (Althaus et al., 2021; Mayer et al., 2011). Causes of insect decline include habitat loss and degradation, for example, arising from intensive agriculture and urbanisation, the use of pesticides, pathogens, the presence of alien species and climate change (Sánchez-Bayo et al., 2019). Pollinator declines may lead to inadequate quantity and quality of pollen reaching the stigmas, and therefore decreasing the sexual reproductive output and compromising yield, termed as pollination deficit (Castro et al., 2021; Garibaldi et al., 2011; Reilly et al., 2020; Woodcock et al., 2019). The abundance of pollinators is important for the delivery of pollination ecosystem services, but the functional divergence between species traits is also considered

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important and maintaining non-overlapping traits distributions could benefit crop pollination and yield (Woodcock et al., 2019). Each bee family is characterised by specific functional traits related to the way they collect pollen and nectar and the way they construct their nests (Danforth et al., 2019; Ropars et al., 2020a) (table 1), and therefore drivers acting at landscape and local habitat scale that impact on the availability of nesting sites and floral resources may impact differently on different functional groups (Graf et al., 2022; Otieno et al., 2015).

The Mediterranean Basin is one of the areas with the highest bee diversity in the world, but this region is also considered as being data deficient (Nielsen et al., 2011; Ropars et al., 2020a; Ropars et al., 2020b). Similarly, despite recent work on Malta's bee diversity and pollination ecosystem services (Balzan et al., 2018a; Balzan et al., 2017; Balzan et al., 2016b), trends relating to bee population sizes, distribution, and conservation remains poorly quantified. Within this context, this study has carried out ecological surveys to develop a preliminary understanding of habitat suitability for honeybees and wild bees, and provide evidence of the role of existing habitats in maintaining pollinators. The following research questions have been addressed:

1. What is the influence of local habitat type on honeybee and wild bee functional group abundance and richness?
2. What is the influence of vegetation community composition on honeybee and bee functional groups abundance and richness?
3. What is the impact of high honeybee visitation on wild bee functional group abundance and richness?

2 Materials and Methods

2.1 Study Area

The study was carried out in the Maltese Islands, a group of low-lying small islands in the Central Mediterranean at 80 km south of Sicily and 241 km east of Tunisia. The Maltese Islands have a Mediterranean climate, characterised by hot and dry summers, and mild and wet winters, having an average total annual rainfall of 543.4 mm for the period 1991–2020, but with high seasonal and inter-annual variability (NSO, 2022). The landscapes of the Maltese Islands have been shaped over several millennia by geo-climatic conditions and long history of human exploitation. Today, around 51% of the territory is characterised by agricultural land use while urban and industrial land uses cover around 30% of the Maltese Islands (Balzan et al., 2016b). Recent research within the study area has provided evidence of a rural-urban gradient in ecosystem service capacities but has also shown significantly high ecosystem service flows to communities in urban green spaces (Balzan et al., 2018a; Balzan et al., 2018b; Balzan et al., 2022). In 2019, Malta had a hive density of 12.86 hives/Km^2. This is significantly higher than the hive density recorded by Chauzat et al. (2013) for other European countries which had an average of 4.2±3 hives/Km^2. A total of 105 bee species have been recorded from the Maltese Islands (Balzan et al., 2016a; Balzan et al., 2017; Cassar et al., 2020) but despite recent research indicating continued pollinator declines in other EU countries and at regional scale (e.g., Bartomeus et al. (2019), Danforth et al. (2019), Dicks et al. (2021) and Vasiliev et al. (2021)), and the growing policy importance (e.g., the EU Pollinators Initiative[1]), there is a limited understanding of the impact of land management and cultural practices on pollinator abundance, diversity and conservation in the Maltese Islands (Balzan et al., 2016b), which observation also applies to other pollinator groups, including but not limited to Lepidoptera, Syrphidae and other pollinating species.

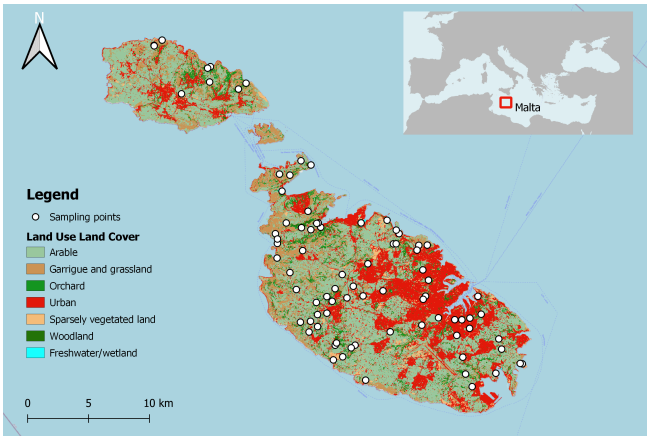

Figure 1: A map showing the location of the sampling points and the land use land cover in the Maltese Islands.

2.2 Sampling strategy and data collection

A total of 78 sites from Malta and Gozo have been surveyed between April and June 2020 (figure 1). A total of 8 habitat types, and namely road verge, garrigue, maquis, garden, steppe, sand dune, woodland, wetland, abandoned field and agriculture fields, have been surveyed to ensure coverage of different habitats and vegetation taxa. The surveys were carried out in dry, cloudless, and low wind conditions, and consisted of timed walks of 20 minutes along standard geolocated belt transects of 2 m×25 m at each site during which flower-visiting bees

[1]EU Pollinators Initiative. Available from: https://ec.europa.eu/environment/nature/conservation/species/pollinators/policy_en.htm. Accessed 19 November 2022.

were caught using a hand net and recorded. Bees which were identified in the transect were released immediately, while specimens collected were subsequently identified to the genus/cf species using identification keys (Michener, 2007) and the reference collection of the first author.

2.3 Functional traits data

Information on the functional traits of wild bees has been gathered through the measurement of the Inter-Tegular Distance (ITD) in mm with a microscale rule. ITD corresponds to the shortest linear distance between a bee's wings at the dorsal side of the thorax which corresponds to the size of wing muscles and to the activity range of a species (Greenleaf et al., 2007). The functional traits associated with nesting type and pollen transport were identified for each specimen based on the literature (table 1).

2.4 Landscape Composition

We obtained land cover data from (Balzan et al., 2018a), who developed a land use land cover (LULC) map using Sentinel 2 satellite images provided by Copernicus (Drusch et al., 2012). As we were interested in assessing the impact of different land cover on bee abundance, we grouped different land uses into the following land cover classes:

1. arable crops,
2. orchards/permanent crops,
3. sparsely vegetated land,
4. garrigue and grassland communities,
5. urban,
6. woodland and
7. wetland/freshwater ecosystems.

The area of different land use land cover (LULC) classes was calculated in buffers with radii of 250 m and 500 m drawn from the sampling points.

2.5 Data Analysis

Generalised Linear Models (GLM) with a Poisson error distribution were used to investigate the influence of habitat and plant diversity on wild bee diversity. A top-down strategy for model selection was used, and the most parsimonious model was selected as that with the lowest Akaike Information Criterion (AIC). GLMs were also used to evaluate the impact of *A. mellifera* abundance on wild bee functional abundance across different habitats. Similarly, the most parsimonious models for each functional wild bee group were selected using a top-down strategy while keeping the model with the lowest AIC. Additionally, a Principal Component Analysis (PCA) was used to investigate the impact of the local habitat type on wild bee diversity. For habitats that were more strongly represented in the dataset ($n > 2$), ordiellipses were created around the sites according to the habitat type with each ellipse representing the 95% confidence interval for the centroid of each group (Oksanen et al., 2019). The impact of landscape habitat composition on *A. mellifera* and wild bee functional group abundance was analysed using zero-inflated Generalised Linear Mixed Models (GLMMs) with Poisson error distribution and with categorical land cover classes as a fixed effect (Fournier et al., 2012; Skaug et al., 2013). To remedy for pseudoreplication arising from sampling in different habitat types, local habitat identity was included as a random variable within the GLMM (Balzan et al., 2016a). Spatial and statistical analyses were carried out using R statistical software (R. Core Team, 2020).

3 Results

During the timed transects, flower visitation was recorded from a total of 74 plant species recorded, from 8 habitat types, amounting to a total of 2610 plant-bee interactions (table 2). Honeybees were the most dominant species in this plant-bee network, performing 86.3% of all interactions. Crown daisy, *Glebionis coronoria*, had the highest visitation rate for wild bees (table 3) but was also one of the most visited flowers by honeybees with *Galactites tomentosa* and *Cynara cardunculus* (figure 3). *G. coronaria* attracted different wild bee groups and species and accounted for 10.2% of all plant-bee interactions and 32.7% of all plant-wild bee interactions. The local habitat type impacted on honeybee abundance (table 3), which was significantly positively associated with agricultural fields but negatively associated with the garden, maquis, road verge and sand dune habitats during our surveys. Increased plant richness was associated with an increased abundance of honeybees in several habitats but negative associations with plant richness were recorded in agricultural fields and garrigue habitats. No significant associations were recorded between the local habitat type, plant richness, and wild bee abundance and richness (table 3). Additionally, no significant association between bee size (ITD) and local habitat type was recorded in this study ($H = 14.37$, $df = 9$, $p = 0.11$). Different functional groups were associated with different habitats, as demonstrated by the PCA (figure 3). Principal Component 1 (PC1) explained a total of 48.5% of the total variance while PC2 explained 32.9% of the variance, and the remaining principal components explained less than 11% each of the remaining variance in the wild bee distribution data. The functional group scores indicate a strong positive association of Legs only, Excavator: Dead Wood and Renter functional groups and a negative association of Legs and Body and Excavator: Ground with PC1. All wild bee functional groups were negatively associated with PC2, but the strongest associations were

Trait category	Variable type	Definition	Taxa
Nesting type	Renter: Existing cavities above-ground	Nesting in pre-existing cavities	*Anthidium* spp.; *Bombus terrestris*; *Megachile* spp.; *Osmia* spp.; *Hylaeus* spp.; *Rhodanthidium* spp.
	Excavator: Ground	Excavating nest in the ground	*Andrena* spp.; *Anthophora canescens*; *Amegilla quadrifasciata*; *Eucera* spp.; *Halictus* spp.; *Seladonia* spp.; *Lassioglossum* spp.; *Panurgus siculus*
	Excavator: Dead Wood	Excavating nest in dead wood	*Xylocopa violaceae*
	Plant stems	Bees which excavate the center of a plant stem to build their nest	*Ceratina* spp.
Pollen Transport	Legs and Body	Pollen is crammed against the inside upper surfaces of their back legs	*Amegilla* spp.; *Andrena* spp.; *Halictus* spp.; *Lassioglossum* spp.; *Seladonia* spp.
	Legs only	Females present scopae which are specialized leg hairs, usually long and sticky	*Anthophora* spp; *Bombus terrestris*; *Ceratina* spp; *Eucera* spp.; *Osmia* spp.; *Panurgus siculus*; *Xylocopa violaceae*;
	Underside: Abdomen	Pollen is carried on long scopal hairs on abdomen	*Anthidium* spp.; *Megachile* spp; *Rhodanthidium* spp.
	Crop	Pollen is carried internally; not presenting external hairs	*Hylaeus* spp.
Body Size	ITD (mm)	The shortest linear distance measured between a wing tegulae across the dorsal thorax	All Taxa

Table 1: Considered wild bee functional traits according to the taxa considered in this study.

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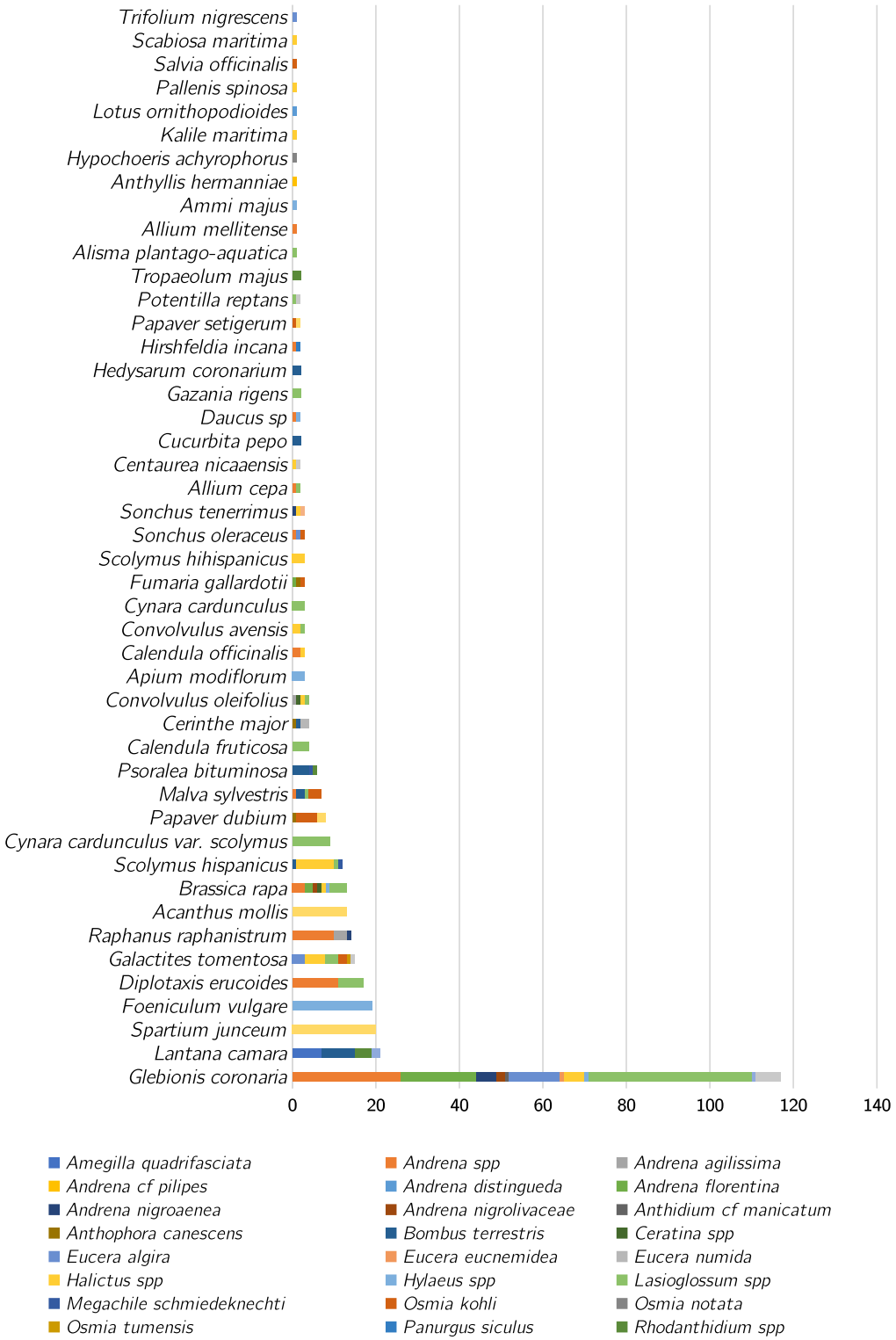

Figure 2: Recorded plant-wild bee interactions.

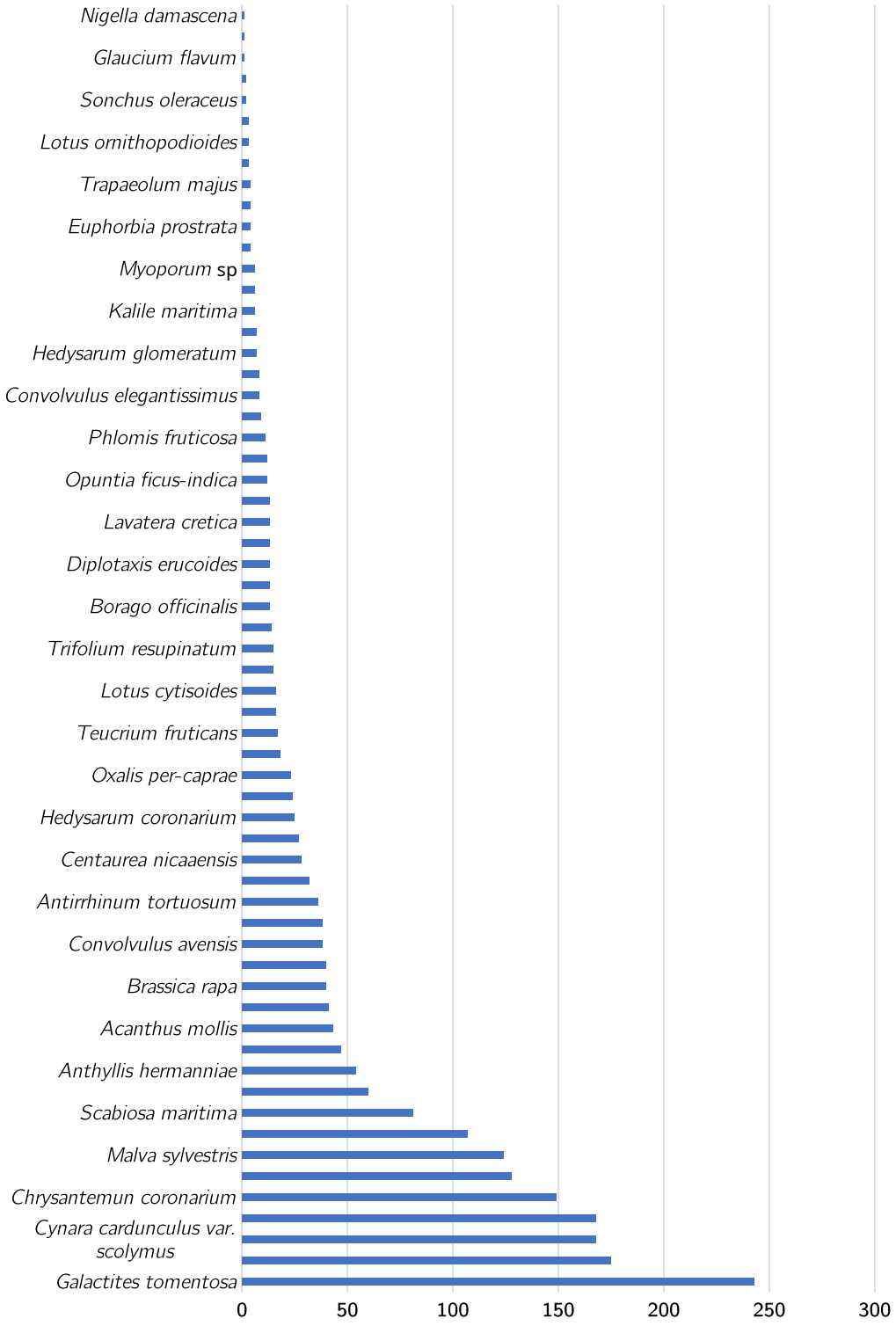

Figure 3: Recorded plant-*A. mellifera* interactions.

recorded with Legs and Body, Legs only, and Excavator: Ground (Appendices). While there is overlap between the ordiellipses, the Excavator: Ground and Legs and Body functional groups were more strongly associated with agricultural field habitats while the strongest association of the other wild bee functional groups was recorded with garden and road verge habitats (figure 4). No significant effect of local habitat type on wild bee functional group richness was recorded but the variation in the distribution of different wild bee groups was also confirmed using GLMs, in which road verge habitats were most strongly associated with the renter nesting type and crop pollen transport functional groups, while the latter was also positively associated with agricultural field habitats (table 4). Garrigue habitats were negatively associated with the Excavator: Ground and Legs and Body functional groups. High honeybee visitation rate had a negative impact on wild bee abundance but no significant impact on pollen collection and nesting type functional group richness was recorded. Significant negative associations between *A. mellifera* visitation rates and the Excavator: Ground, Legs and Body, and Crop functional groups abundance were recorded. For the Excavator: Ground and Legs and Body functional groups positive interactions between *A. mellifera* abundance and agricultural field, garrigue and sand dune habitats were recorded. No significant associations with habitat type and *A. mellifera* abundance were recorded for the Excavator: Dead Wood, Legs Only, and Underside: Abdomen functional groups while the models were not run with the Plant functional group given the low abundance of *Ceratina sp.* ($n = 2$). At both landscape scales considered in this study (r=250 and 500 m), agricultural (arable and orchard) and urban land cover were positively associated with the abundance of renter nesting type and crop pollen collection functional groups (table 5). Similarly, a positive association between arable and urban land cover and the legs-only pollen collection functional group was recorded in the landscape buffers with a radius of 500 m. Sparsely vegetated land and woodland were positively associated with *A. mellifera* but were negatively associated with the Excavator: Ground functional.

4 Discussion

4.1 Impact of local habitat conditions on bee abundance

Honeybee abundance was positively associated with agricultural habitats while, when all wild bee functional groups are considered, there was no impact of local habitat type on wild bee abundance and diversity (table 3). Previous research indicates that wild bees tend to be more abundant on flowers of wild-growing plants than on cultivated ones while honeybees prefer mass flowering crops (Herrera, 2020; Rollin et al., 2013). In this study, honeybees used a diverse range of floral resources (figure 3) and while there is substantial overlap with the plant species list developed by Balzan et al. (2018a) through stakeholder engagement, several additional species are recorded here indicating a wider use of floral resources by honeybees. Results presented here also support previous observations obtained through expert ranking with beekeepers from the Maltese Islands, in which agricultural fields were considered the most important habitats for honeybees in spring (Balzan et al., 2018a). In line with observations of higher flower constancy in honeybees when compared to solitary bees (Rollin et al., 2013; van der Niet et al., 2020), a negative association of honeybee abundance with plant richness was recorded in agricultural habitats. However, in our study, this trend was reversed in habitats with a lower abundance of honeybees, namely garden, road verge, maquis and sand dune habitats in which honeybee abundance was positively associated with plant richness. Overall, there was no impact of local habitat type or plant richness on wild bee abundance or diversity (table 3), but our findings suggest that wild bees and honeybees differ in their patterns of habitat and floral resource use at the local scale. There was also variation in the use of different habitats between different wild bee functional groups (table 4). The PCA analysis distinguishes the Excavator: Ground nesting and Legs and Body pollen collection functional groups as being mostly associated with agricultural fields, but this association was not recorded in the GLM analysis which also considered the impact of *A. mellifera* abundance. The renter nesting type and crop pollen transport were positively associated with road verge habitats in the GLM analysis, supporting observations that spontaneous roadside vegetation can act as a hotspot of flowering plants within the landscape and are important to support wild bee diversity (Heneberg et al., 2017; Hopwood, 2008; Phillips et al., 2020).

4.2 Evidence of competition between honeybees and wild bees

High honeybee visitation rate had a negative impact on wild bee abundance but no significant impact on functional group richness was recorded in this study. These results provide the first evidence of the impact of honeybee abundance on wild bees in the Maltese Islands and are in line with correlative and experimental evidence from the Mediterranean region, which has shown that at local and regional scales, honeybees have strong negative impacts on wild bee populations and leading to predictions of a gradual long-term replacement of wild bees by honeybees in flowers (Herrera, 2020). Since honeybees occupy central positions in plant-pollinator networks (Lázaro et

Surveyed habitats	No of sites	Plants recorded
Road Verge	21	*Acanthus mollis; Antirrhinum tortuosum; Borago officinalis; Brassica rapa; Calendula fruticose; Centaurea nicaeensis; Cerinthe major; Glebionis coronaria ; Convolvulus arvensis; Convolvulus oleifolius; Daucus gingidium; Euphorbia prostrata; Foeniculum vulgare; Galactites tomentosa; Hirschfeldia incana; Lantana camara; Lavatera cretica; Lotus cytisoides Malva sylvestris; Nigella damascene; Oxalis pes-caprae; Papaver dubium; Pallenis spinosa; Prasium majus; Psoralea bituminosa; Scabiosa maritima; Sonchus oleraceus; Sonchus oleraceus ; Teucrium flavum; Trifolium nigrescens;*
Garden	18	*Acanthus mollis; Ammi majus; Antirrhinum tortuosum; Borago officinalis; Brassica rapa; Calendula officinalis; Calendula fruticose; Cheirolophus crassifolius; Glebionis coronaria; Convolvulus elegantissimus; Convolvulus oleifolius; Diplotaxis erucoides; Fumaria gaillardotii; Galactites tomentosa; Gazania rigens; Glaucium flavum; Hedysarum coronarium; Helichrysum melitense; Hypochoeris achyrophorus; Lantana camara; Lavatera cretica; Lotus ornithopodioides; Malva sylvestris; Myoporum; Ononis natrix subsp. Ramosissima; Origanum majorana; Papaver dubium; Papaver setigerum; Phlomis fruticose; Raphanus raphanistrum; Spartium junceum; Sonchus tenerrimus Tropaeolum majus; Spartium junceum; Thymbra capitate, Teucrium fruticans*
Agriculture Field	12	*Allium cepa; Cynara cardunculus scolymus; Cucurbita pepo; Papaver dubium; Apium nodiflorum; Brassica rapa; Glebionis coronaria; Convolvulus arvensis; Diplotaxis erucoides; Spartium junceum; Potentilla reptans*
Abandoned Field	12	*Alisma plantago-aquatica; Brassica rapa; Centaurea nicaeensis; Glebionis coronaria; Fumaria gaillardotii; Galactites tomentosa; Hedysarum glomeratum; Malva sylvestris; Mentha pulegium; Papaver dubium; Papaver setigerum; Raphanus raphanistrum; Salvia officinalis; Scabiosa maritima; Teucrium fruticans; Teucrium flavum; Trifolium resupinatum; Trifolium nigrescens; Tropaeolum majus*
Garrigue	10	*Allium melitense; Anthyllis hermanniae; Brassica rapa; Glebionis coronaria; Centaurea nicaeensis; Cynara cardunculus; Convolvulus oleifolius; Foeniculum vulgare; Galactites tomentosa; Hedysarum glomeratum; Lotus ornithopodioides; Malva sylvestris; Psoralea bituminosa; Raphanus raphanistrum; Teucrium fruticans; Thymbra capitata*
Sand dunes	4	*Cakile maritima; Centaurea nicaeensis; Cynara cardunculus; Convolvulus oleifolius; Galactites tomentosa; Lotus cytisoides; Scolymus hispanicus; Scabiosa maritima; Tamarix africana*
Maquis	2	*Cerinthe major; Cercis siliquastrum; Glebionis coronaria; Galactites tomentosa; Psoralea bituminosa; Sonchus tenerrimus*
Steppe	2	*Daucus gingidium; Galactites tomentosa; Lotus cytisoides; Lotus ornithopodioides; Oxalis pes-caprae; Psoralea bituminosa*
Wetland	2	*Ononis natrix* subsp. *Ramosissima; Tamarix africana*
Woodland	2	*Convolvulus arvensis; Glebionis coronaria; Galactites tomentosa; Opuntia ficus-indica;*

Table 2: Plants recorded within the different habitats.

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Explanatory variables	(a) *A. mellifera* abundance			(b) Wild bee abundance			(c) Wild bee richness		
	Est (±SE)	Z-val	p-val	Est (±SE)	Z-val	p-val	Est (±SE)	Z-val	p-val
Intercept	2.91(0.18)	15.80	<0.001	1.51(0.39)	3.84	<0.001	1.04(0.43)	2.41	0.02
Agricultural fields	3.05(0.22)	13.60	<0.001	0.53(0.61)	0.88	NS	0.04(0.81)	0.06	NS
Garden	-0.21(0.2)	-1.03	NS	-0.25(0.44)	-0.57	NS	0.06(0.50)	0.13	NS
Garrigue	-1.11(0.23)	4.80	<0.001	0.51(0.56)	0.91	NS	-0.35(0.67)	-0.53	NS
Maquis	-3.50(1.03)	-3.39	<0.001	-1.50(1.55)	-0.97	NS	0.79(1.11)	0.71	NS
Road verge	-0.48(0.22)	-2.18	0.03	-0.02(0.46)	-0.05	NS	0.25(0.51)	0.49	NS
Sand dunes	-0.61(0.28)	-2.16	0.03	0.46(0.61)	0.74	NS	0.43(0.70)	0.62	NS
Steppe	-0.22(0.51)	-0.43	NS	14.80(2103)	0.01	NS	1.16(1.59)	0.73	NS
Wetland	-0.41(0.51)	-0.80	NS	-17.80(4703)	-0.004	NS	-1.04(2.28)	-0.46	NS
Woodland	-0.19(0.46)	-0.41	NS	-27.11(3155)	-0.01	NS	-1.59(1.59)	-1.00	NS
Plant richness	0.01(0.05)	0.22	NS	-0.02(0.12)	-0.15	NS	0.06(0.20)	0.47	NS
Agricultural fields:									
Plant richness	-0.94(0.08)	-11.16	<0.001	0.06(0.20)	0.29	NS	0.12(0.26)	0.47	NS
Garden:									
Plant richness	0.22(0.06)	3.77	<0.001	0.15(0.13)	1.16	NS	-0.01(0.14)	-0.07	NS
Garrigue:									
Plant richness	-0.2(0.07)	-2.71	0.007	-0.22(0.19)	-1.16	NS	0.05(0.20)	0.29	NS
Maquis:									
Plant richness	0.98(0.27)	3.59	<0.001	0.36(0.45)	0.81	NS	-0.17(0.36)	-0.48	NS
Road verge:									
Plant richness	0.22(0.06)	3.50	<0.001	0.03(0.14)	0.23	NS	-0.04(0.15)	-0.32	NS
Sand dunes:									
Plant richness	0.43(0.07)	5.53	<0.001	-0.16(0.21)	-0.76	NS	-0.14(0.22)	-0.64	NS
Steppe:									
Plant richness	0.17(0.15)	1.09	NS	-8.13(1051)	-0.01	NS	-0.61(0.59)	-1.03	NS
Wetland:									
Plant richness	0.49(0.29)	1.692	0.09	0.01(2974)	0.00	NS	-0.06(1.42)	-0.04	NS
Woodland:									
Plant richness	-0.9(0.2)	-0.44	NS	9.32(1051)	0.01	NS	0.49(0.59)	0.83	NS
AIC	1574.54			478.65			307.88		
ΔAIC	-427.33			-5.90			-176.67		

Table 3: Parameter estimates for bee abundance using GLMs with a Poisson error distribution and according to the local habitat type and plant richness.

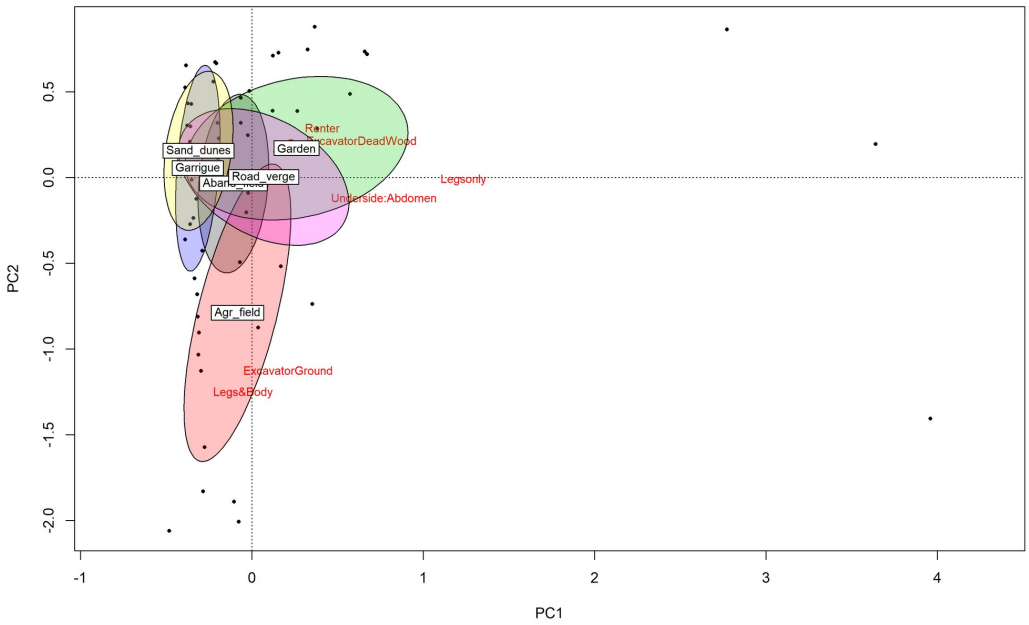

Figure 4: Principal Component Analysis (PCA) of wild bee functional group abundance according to local habitat type.

al., 2021; Petanidou et al., 2008; Ropars et al., 2022), a high abundance of honeybees may also affect the structure of plant-pollinator interactions and the functioning of ecosystems (Geslin et al., 2017). Honeybees reduce pollen and nectar availability, competitively displace wild pollinators from floral resources and influence their foraging behaviour, and the probability of observing wild bee foraging on wildflower patches was found to drop significantly when honeybees are present (Lázaro et al., 2021; Ropars et al., 2022; Shavit et al., 2009). Our findings indicate a significant negative impact of the high honeybee visitation rate on the Excavator: Ground nesting type and the Legs and Body and Crop pollen collection, which as documented by the PCA were associated with the agricultural field habitat type. As documented in previous research assessing the contribution of different ecosystems to pollination ecosystem services and honey production (Balzan et al., 2018a), honeybee flower visitation is positively correlated with agricultural field habitats (table 4). The strongest effect is therefore recorded for wild bees having similar ecological requirements (Lázaro et al., 2021; Ropars et al., 2022), with previous research indicating that the competition effect is highest around the apiaries and spanned distance of 600-1100 m around apiaries (Henry et al., 2018). Additionally, the impact of honeybee visitation on wild bee communities would be expected to be higher in habitat patches having lower availability of floral resources (Rodríguez et al., 2021).

4.3 Impact of landscape composition on honeybee and wild bee abundance

The landscape composition impacted the honeybee and wild bee functional group visitation rate. Honeybee visitation is influenced by the location of the apiaries which showed a positive association with woodland and garrigue and grassland habitats but were negatively associated with urban land use cover in Balzan et al. (2018a). The abundance of bees in the renter and crop functional group categories increased with agricultural and urban land cover, which contrasts with common observations that more intensive land uses are associated with species loss. For example, Clough et al. (2014) recorded reduced density of plants depending on bee and insect pollination with increasing arable land use, while lower bee species richness and abundance were recorded with a high intensity of farmland management (Hendrickx et al., 2007) and in residential areas compared to urban fringe areas (Mcintyre et al., 2001). The benefit from higher land use intensity, in the form of agricultural and urban land uses, may be explained by the increased availability of nesting sites caused by man-made structures (e.g., rubble walls and buildings) or may have been due to lower interspecific competition (Klein et al., 2002). Our findings provide evidence of interspecific competition arising from high *A. mellifera* (Evidence of competition between honeybees and wild bees) visitation in agricultural habitats while pre-

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Explanatory variables	(a) Wild bee			(b) Renter			(c) Excavator Ground			(d) Legs & Body			(e) Crop		
	Est (±SE)	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**
Intercept	1.93(0.21)	9.16	<0.001	-0.58(0.42)	-1.38	NS	1.77(0.23)	7.73	<0.001	2.02(0.24)	8.45	<0.001	-0.73(0.72)	-1.01	NS
Agricultural fields	0.3(0.29)	1.06	NS	0.59(6.50)	0.91	NS	0.11 (0.32)	0.33	NS	-0.18(0.33)	-0.36	NS	1.76(0.87)	2.03	0.04
Garden	-0.45(0.26)	-1.72	0.08	0.88(0.47)	1.89	0.06	0.15(0.32)	0.49	NS	-0.3(-0.34)	-0.90	NS	1.49(0.79)	1.88	0.06
Garrigue	-0.53(0.3)	-1.74	0.08	-1.54(1.08)	-1.43	NS	-1.76(0.45)	-3.91	<0.001	-0.75(-0.33)	-2.25	0.02	-16.39(1940)	-0.01	NS
Maquis	-1.35(0.85)	-1.59	NS	0.68(0.82)	0.83	NS	-1.95(1.19)	-1.64	0.10	0.58(336.5)	0.00	NS	-17.42(5927)	-0.003	NS
Road verge	-3.1(0.27)	-1.18	NS	1.39(0.44)	3.18	0.001	-0.41(-0.30)	-1.37	NS	-0.42(-0.30)	-1.44	NS	1.84(0.74)	2.47	0.01
Sand dunes	-0.25(0.39)	-0.63	NS	0.12(0.82)	0.14	NS	67.42(4992)	-0.01	NS	-0.4(0.42)	-0.95	NS	-16.15(2370)	-0.01	NS
Steppe	36.11(4908)	0.01	NS	15.56(1486)	-0.01	NS	40.93(13340)	0.00	NS	36.02(4908)	0.00	NS	-16.45(6556)	-0.00	NS
Wetland	-18.24(6243)	-0.00	NS	15.56(1484)	-0.01	NS	-20.08(16970)	-0.00	NS	18.33(6243)	-0.00	NS	16.41(6442)	-0.00	NS
Woodland	112(12620)	0.01	NS	-15.64(1487)	-0.01	NS	123.4(34310)	0.00	NS	111.9(12620)	0.01	NS	-17.48(6660)	-0.00	NS
A. mellifera abundance	-0.03(0.01)	-2.61	0.009	-0.01(0.00)	-1.26	NS	-0.03(0.01)	-2.45	0.01	-0.08(0.02)	-3.91	<0.001	-0.08(0.02)	-4.68	<0.001
Agricultural fields: *A. mellifera*	2.8(0.01)	2.37	0.02				0.03(0.01)	2.33	0.01	0.08(0.02)	3.88	<0.001			
Garden: *A. mellifera*	0.04(0.01)	3.03	0.002				-0.01(0.01)	0.61	NS	0.04(0.02)	1.77	0.08			
Garrigue: *A. mellifera*	0.03(0.01)	2.37	0.02				0.04(0.01)	3.01	0.002	0.07(0.02)	3.76	<0.001			
Maquis: *A. mellifera*	0.06(0.04)	1.58	NS				0.07(0.05)	1.56	NS	-0.58(84.13)	-0.01	NS			
Road verge: *A. mellifera*	0.03(0.01)	2.01	0.04				0.01(0.01)	1.01	NS	0.06(0.02)	2.76	0.006			
Sand dunes: *A. mellifera*	0.03(0.01)	1.9	0.06				0.08(57.38)	0.01	NS	0.07(0.02)	3.23	0.001			
Steppe: *A. mellifera*	-1.78(233)	-0.01	NS				-2.00(63.53)	-0.00	NS	-1.73(233.7)	-0.01	NS			
Wetland: *A. mellifera*	299.5(229)	0	NS				0.03(622)	0.00	NS	0.08(228.8)	0.00	NS			
Woodland: *A. mellifera*	-9.27(1052)	-0.01	NS				-10.22(2859)	-0.00	NS	-9.23(1052)	-0.01	NS			
AIC				269.7			362.05			420.94	172.43				
ΔAIC				-11.72			-33.16			-36.7	-3.1				

Table 4: Parameter estimates using GLMs with a Poisson error distribution for wild bee functional group abundance data according to the local habitat and honeybee abundance.

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Explanatory variables	(a) *A. mellifera*			(b) Renter			(c) Excavator Ground			(d) Legs and Body			(e) Legs only			(f) Crop		
R = 250m	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**±**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**
Intercept	3.16 (0.26)	12.07	<0.001	-6.97 (2.86)	-2.44	0.01	1.28(0.72)	1.78	NS	1.26(0.65)	1.93	0.05	-1.7(2.42)	-0.7	NS	-20.22(9.43)	-2.15	0.03
Garrigue and grassland	0.00 (0.02)	0.03	NS	0.19 (0.18)	1.07	NS	-0.02(0.04)	-0.5	NS	-0.04(0.05)	-0.83	NS	0.1(0.13)	0.75	NS	1.4(0.66)	2.11	0.04
Arable	-0.05 (0.01)	-4.5	NS	0.37 (0.14)	2.60	0.01	0.06(0.04)	1.56	NS	0.06(0.03)	1.87	0.06	0.08(0.12)	0.63	NS	0.74(0.39)	1.87	0.06
Orchards	0.01 (0.02)	0.32	NS	0.52 (0.18)	2.92	0.004	-0.06(0.06)	-1.04	NS	-0.004(0.06)	-0.08	NS	0.17(0.15)	1.08	NS	1.36(0.62)	2.2	0.03
Sparsely vegetated	0.18 (0.03)	6.27	<0.001	0.34 (0.22)	1.52	NS	-0.37(0.14)	-2.59	0.01	-0.12(0.15)	-0.83	NS	0.03(0.17)	0.15	NS	1.82(1.01)	1.81	0.07
Urban	0.02 (0.01)	1.42	NS	0.42 (0.15)	2.83	0.005	-0.001(0.04)	-0.01	NS	-0.05(0.05)	-1.03	NS	0.23(0.12)	1.86	0.06	1.12(0.47)	2.37	0.02
Woodland	0.22 (0.06)	3.77	<0.001	-0.30 (0.39)	-0.77	NS	0.05(0.18)	0.26	NS	-0.01(0.17)	-0.06	NS	-0.85(0.4)	-2.11	0.04	-0.53(1.13)	-0.47	0.64
R = 500m	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**	**Est (±SE)**	**Z-val**	**p-val**
Intercept	2.94 (0.31)	9.63	<0.001	-5.02 (2.15)	-2.34	0.02	0.84(0.8)	1.05	NS	1.36(0.73)	1.86	0.06	-3.12(1.52)	-2.04	0.04	-16(8.55)	-1.87	0.06
Garrigue and grassland	0.01 (0.00)	1.83	0.06	0.04 (0.04)	0.96	NS	0.01(0.01)	0.91	NS	-0.01(0.01)	-0.49	NS	0.04(0.03)	1.66	0.10	0.33(0.2)	1.67	0.1
Arable	-0.001 (0.003)	-0.35	NS	0.06 (0.03)	2.38	0.02	0.02(0.01)	2.02	0.04	0.01(0.01)	1.91	0.06	0.04(0.02)	2.19	0.03	0.07(0.07)	0.92	NS
Orchards	0.005 (0.005)	0.92	NS	0.12 (0.05)	2.65	0.008	-0.03(0.02)	-1.48	NS	-0.01(0.01)	-0.55	NS	0.05(0.03)	1.54	NS	0.45(0.21)	2.15	0.03
Sparsely vegetated	0.04 (0.01)	3.34	0.001	0.07 (0.06)	1.27	NS	-0.09(0.04)	-2.18	0.03	-0.03(0.03)	-0.9	NS	-0.01(0.06)	-0.22	NS	0.58(0.34)	1.72	0.09
Urban	0.001 (0.003)	0.17	NS	0.08 (0.03)	2.9	0.004	0.004(0.01)	0.38	NS	-0.02(0.01)	-1.5	NS	0.08(0.02)	4.11	<0.001	0.22(0.1)	2.09	0.04
Woodland	0.05 (0.02)	2.89	0.004	-0.14 (0.12)	-1.13	NS	0.04(0.05)	0.63	NS	-0.002(0.05)	-0.03	NS	-0.4(0.16)	-2.57	0.01	-0.35(0.49)	-0.73	NS

Table 5: Parameter estimates using GLMMs with a Poisson error distribution for bee abundance data according to the landscape habitat area in buffers with radii of 250m and 500m.

vious research indicates that apiaries tend to be located away from urban environments (Balzan et al., 2018a). A negative impact of sparsely vegetated land on the Excavator: Ground functional group. This is not surprising given that these spaces would be expected to have reduced soil cover and therefore offer lower nesting habitat availability for these species. Additionally, woodland had a negative impact on the Legs Only functional group which may be associated with lower diversity of foraging plants in understories and therefore may not support bee species (Tscheulin et al., 2011).

4.4 Limitations and recommendations for habitat management for honeybee and wild bee conservation

This study has provided a first analysis of the impact of local and landscape habitats on the abundance and diversity of bees in the Maltese Islands, but such an analysis also remains missing for other insect pollinator groups (e.g., butterflies, moths and hoverflies). Our findings are relevant for the identification of habitat management practices that give rise to benefits to beekeeping practices and ensuring the conservation of the endemic Maltese honeybee *A. mellifera ruttneri* (Sheppard et al., 1997), while also prioritising the conservation of wild bees. However, a key limitation of this study is the spatiotemporal representation of the habitat-plant-bee interactions within the study area. With sampling in our work being carried out in spring and given the varying availability of floral resources in the different habitats of the Maltese Islands (Balzan et al., 2018a), interspecific interactions associated with floral resource use and competition would be expected to vary accordingly while, for example, interspecific competition could increase in periods having lower availability of floral resources, most notably in summer. Our results provide evidence that honeybees and wild bees differ in their patterns of habitat and floral resource use at the local scale and are impacted differently by land use area at the landscape scale. Results from this study indicate a strong association of *A. mellifera* with agricultural habitats, which were also associated with increased abundance of the Excavator: Ground nesting and Legs and Body pollen collection functional groups. Consequently, measures that increase floral resources and nesting habitat availability within this habitat would be expected to lead to benefits for a wider range of wild bee groups and species. Particularly, measures that increase plant diversity contribute more significantly to the maintenance of wild bee diversity in agricultural habitats. We have also shown that road verge habitats provide important floral resources and increased the abundance of the renter and crop functional groups. During the data collection, we noticed that roadside vegetation continued providing floral resources even after the removal of crops in agricultural fields at the start of the dry season, and the management of road verge habitats to ensure their persistence within the landscape would be expected to lead to benefits to bee communities. Reducing the frequency of mowing to 0–2 cuts/year allows wildflowers and larval foodplants for other pollinator groups to grow and reduces the risk of direct mortality to pollinators (Phillips et al., 2020). We also provide the first evidence of competition between honeybees and the wild bees of the Maltese Islands. Given the high cultural value of endemic honeybee subspecies and beekeeping, policy action has often focused on providing floral resources for honeybees and supporting beekeeping. While further research is needed, findings presented here provide evidence that the use of floral resources varies according to wild bee functional group, and measures such as the establishment of monospecific nitrogen-fixing crops (e.g., sulla *Hedysarum coronarium*) as part of the crop rotation would not necessarily translate to increased provision of floral resources to other wild bee groups, especially within the context of the negative honeybee-wild bee interactions. Measures that lead to further beekeeping intensification through unplanned increases of apiaries, for example as part of agri-environment-climate measures, for pollination ecosystem services or honey production, may be counterbalanced by the loss of the wild bees and their contribution to pollination services. Further research is, therefore, needed to model the distribution of different wild bee species and understand how these interact with varying honeybee density across the Maltese Islands, and identify pollination deficits in crops and wild plants. The analysis of plant-pollinator interaction networks can shed further light of the impacts of beekeeping intensification on pollinator diversity and pollination ecosystem services. Higher hive density also impacts on honeybee health by making it easier for pathogens to spread (le Conte et al., 2010), suggesting the need for further research that assesses the implications of high hive densities on honeybee health.

5 Conclusions

This study has carried out a preliminary assessment of the local and landscape habitat variables impacting on honeybees and wild bee populations in the Maltese Islands. First, our findings indicate that honeybees were most strongly associated with agricultural habitats and used a wide diversity of floral resources, which overlapped with those used by wild bees. Second, at the local scale, no significant effect of local habitat type on wild bee functional richness was recorded but we observed variation in flower visitation by different wild bee functional groups. Excav-

ator: Ground nesting and Legs and Body pollen collection were associated with agricultural fields while the other bee functional groups increased in road verge and garden habitats. Third, at the landscape scale, the abundance of bees in the renter and crop functional group categories increased with agricultural and urban land cover, which may be a consequence of reduced interspecific competition or increased availability of nesting sites. Fourth, our results indicate that honeybee abundance was negatively related to the abundance of wild bees, with negative impacts of the high honeybee visitation rate being recorded for functional groups associated with agricultural habitats. We identify the need for further research to evaluate the impacts of honeybee competition across spatial and temporal scales. These results demonstrate that measures that lead to increasing hive density for pollination ecosystem service or honey production may be counterbalanced by the loss of wild bees and impacts on bee health.

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Appendices

Principal Component Analysis species and site scores

	PC1	PC2	PC3	PC4	PC5	PC6
Renter	0.841	−0.566	0.355	−0.560	−0.426	−0.240
Excavator: Ground	−1.198	−2.237	1.414	1.002	−0.029	−0.007
Excavator: Dead Wood	1.406	−0.575	−1.473	1.151	−0.209	0.021
Plant	−0.005	−0.005	0.009	−0.025	−0.100	0.019
Legs and Body	−2.823	−2.471	−1.121	−0.597	0.019	−0.004
Legs only	3.597	−2.268	0.037	−0.393	0.222	0.000
Underside: Abdomen	0.113	−0.149	0.085	−0.148	0.034	0.019
Crop	0.444	−0.439	0.361	−0.454	−0.291	0.333

Principal Component Analysis Functional Group (species) scores.

	PC1	PC2	PC3	PC4	PC5	PC6
sit1	−0.139	−0.205	0.705	0.600	1.233	0.206
sit2	−0.075	0.333	0.399	0.300	0.742	0.312
sit3	0.006	0.431	0.213	−0.162	−0.327	−1.062
sit4	0.610	0.192	0.875	0.138	1.280	−1.047
sit5	0.149	1.147	0.041	−0.121	0.277	0.485
sit6	0.149	1.147	0.041	−0.121	0.277	0.485
sit7	0.467	0.324	0.868	0.255	0.684	−1.049
sit8	0.405	0.554	0.676	−0.091	−0.981	−2.425
sit9	0.244	0.883	0.296	0.060	0.795	0.446
sit10	−0.075	0.333	0.399	0.300	0.742	0.312
sit11	−0.139	−0.205	0.705	0.600	1.233	0.206
sit12	0.037	1.002	−0.156	−0.297	0.328	0.460
sit13	−2.200	−1.885	−4.095	−3.826	1.355	−0.042
sit14	−0.597	−0.287	0.424	0.179	−1.786	0.699
sit15	−0.011	0.872	0.092	−0.001	0.250	0.418
sit16	−1.444	−1.603	0.556	1.079	0.011	−0.182
sit17	0.149	1.147	0.041	−0.121	0.277	0.485
sit18	0.182	0.266	0.282	−0.444	−0.878	−2.476
sit19	−0.807	−0.503	0.350	0.599	0.117	0.085
sit20	−0.489	0.047	0.247	0.359	0.170	0.218
sit21	−0.024	0.799	−0.227	−0.773	−1.550	0.982
sit22	−0.123	0.727	−0.105	−0.177	0.302	0.393
sit23	−0.170	0.597	0.144	0.119	0.224	0.352
sit24	0.037	1.002	−0.156	−0.297	0.328	0.460
sit25	1.338	0.150	−1.474	1.224	0.473	1.257
sit26	0.196	0.429	−0.028	−0.582	0.078	−0.908
sit27	−0.170	0.597	0.144	0.119	0.224	0.352
sit28	1.293	0.151	−0.831	0.212	−0.695	−1.828
sit29	−1.565	−2.320	0.406	1.543	−0.010	−0.187
sit30	0.347	0.981	−0.211	0.104	0.310	0.614
sit31	−0.329	0.322	0.195	0.239	0.197	0.285
sit32	−0.299	0.569	−0.747	−0.826	0.482	0.385
sit33	0.149	1.147	0.041	−0.121	0.277	0.485
sit34	−0.592	−1.110	0.218	0.661	−0.428	−1.267
sit35	0.347	0.981	−0.211	0.104	0.310	0.614
sit36	−0.708	−0.050	−1.457	−1.741	−0.368	−1.018
sit37	−0.915	−0.656	0.168	0.379	0.260	0.173
sit38	−0.967	−0.778	0.401	0.719	0.091	0.018
sit39	−0.760	−0.373	0.101	0.303	0.195	0.127
sit40	3.672	−1.842	−3.860	2.902	−0.302	−0.284
sit41	−1.078	−0.923	0.204	0.543	0.142	−0.007
sit42	−0.329	0.322	0.195	0.239	0.197	0.285
sit43	−1.509	−2.142	0.862	1.379	0.502	−0.288
sit44	0.149	1.147	0.041	−0.121	0.277	0.485
sit45	0.676	0.650	0.248	−0.966	−1.376	−3.756
sit46	0.674	−0.126	−0.740	0.716	−1.575	1.413
sit47	3.761	−3.516	2.852	−3.168	0.938	0.922
sit48	0.946	−0.041	0.383	−1.297	−0.034	1.525
sit49	−0.465	−0.682	0.487	−0.720	−5.266	1.880

Principal Component Analysis site scores.

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Review Article

From Click Chemistry to Fluorescent Molecular Logic Gates

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Abstract. The 2022 Nobel Prize in Chemistry was awarded for the inventions of click chemistry and biorthogonal chemistry, which culminated from trans-disciplinary research in the areas of organic synthesis, fluorescence imaging and chemical biology. In this mini-review, the inventions of the Pourbaix sensor and the lab-on-a-molecule, both examples of purposely designed and synthesised functional molecules with fluorescence properties, are discussed. These intelligent molecules operate on the premise of a competitive tension between non-radiative photoinduced electron transfer (PET), and radiative fluorescence. Redox, acid-base and ion-binding equilibria modulate the states of these molecular devices. Potential applications of these inventions in corrosion detection, cell imaging and health diagnostics for the benefit of society are presented.

Keywords: click chemistry, triazole, molecular logic gates, photoinduced electron transfer, fluorescence, Pourbaix sensor.

1 An Introduction to Click Chemistry

The 2022 Nobel Prize in Chemistry was awarded for the invention of "click chemistry and biorthogonal chemistry" (Kolb et al., 2001; Prescher et al., 2005). It is a class of reaction that joins two molecular entities together in a predictable manner to produce a single product in high yield. This is the gold standard by chemists performing organic reactions. Two of the three winners, Morten Meldal of the University of Copenhagen, and Barry Sharpless of Scripps Research in La Jolla, California, developed the chemistry independently, working away on different problems. Sharpless was thinking about developing more efficient synthetic reactions (Kolb et al., 2001). Meldal was developing organic reactions that could be run on solid-phase peptide synthesis supports for drug discovery (Tornøe et al., 2002; Tornøe et al., 2004). They both demonstrated an efficient way of reacting an azide (a chemical group with three nitrogen atoms) and an alkyne (a chemical group with two carbon atoms connected by a triple bond) to form a triazole (a five-membered ring of two carbon and three nitrogen atoms). This chemistry was previously known as the Huisgen 1,3-dipolar [2+3] cycloaddition (Huisgen et al., 1967), but the reaction was less than ideal. The reactions required heating, and took hours or days, and the yields were generally poor with a mixture of 1,4 and 1,5 isomers. The Copenhagen and La Jolle teams discovered that the presence of trace copper ions greatly speeds up the reaction without the need for heating and the result is formation of only the 1,4-isomer. The reaction is stereospecific and selective for the formation of a single product.

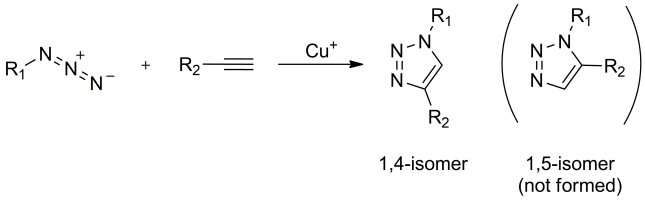

Figure 1: The 1,3-dipolar Cu(I) catalysed click reaction between an azide and an alkyne forming the 1,4-triazole product (and not the 1,5-triazole).

However, it was the trans-disciplinary aspect, the application of this chemistry to the field of chemical biology, that demonstrated the usefulness of this invention. Within living systems, alkyne and azide groups are not found. It so happens that these functional groups are generally non-reactive and non-toxic to living things. The third award winner, Carolyn Bertozzi of Stanford University, developed a copper-free "click" reaction, as copper(I) is toxic to living cells. The goal of the Bertozzi group was to use fluorescent reporter molecules that could attach to polymeric sugar molecules found on the surface of living cells called glycans, which are large carbohydrate

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molecules located on the surface of cells. She named her invention biorthogonal chemistry, which refers to any chemical reaction that can occur inside living systems without interfering with the native biochemical cellular processes (Prescher et al., 2005). Then using a fluorescence microscope, the site of the bond formed with the fluorescent reporter can be observed and the target of interest located and quantified. The approach has been expanded to the specific labelling of cellular proteins and studying of drug targets in live cells (Kim et al., 2019). The copper-free click reaction uses a strained alkyne, such as cyclooctyne (Agard et al., 2006). Alkynes favour a linear geometry. However, in cyclooctyne the two single carbon-carbon bonds on either side of the alkyne are bent, turned inward within the eight-membered carbon ring. In this form, the cycloalkyne is under strain. Because of the pent-up strain energy, there is no need for a catalyst. The relieve of the strain energy from the compressed geometry during the chemical transformation from a triple bond (alkyne) to a double bond (alkene) on formation of the triazole ring provides the reaction with the thermodynamic driving force necessary to overcome the activation barrier. Figure 2 illustrates this approach to a click reaction.

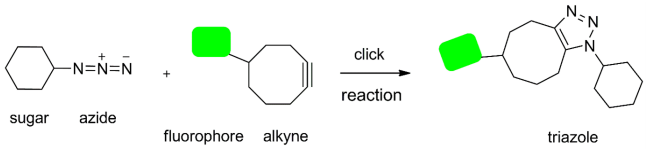

Figure 2: The click reaction between a generic sugar modified with an azide, and a fluorophore-labelled cyclooctyne.

The Bertozzi group applied this chemistry to living cells and animals. Modified sugar molecules with the azide group were fed to the living cells. The cells assimilated the modified sugar molecules as if they were no different than the unmodified sugar molecules and incorporated them into the glycans. Once the modified sugars were stationed on the extremity of the cell within the glycan structure, addition of a (fluoro-labelled) cyclooctyne resulted in the click chemistry on the cell interface. The alkyne reacts with the azide to form two strong chemical bonds resulting in the triazole product figure 2. The location of the glycans are then trackable by a fluorescence signal from the result of the click reaction on irradiation of the cells with light.

2 An Introduction to Fluorescent Indicators and Pourbaix Sensors

The first explicit report of fluorescent pH indicators was with the polyaromatic hydrocarbon anthracene as the fluorescent reporter (de Silva et al., 1985). Fluorescent redox (pE) indicators soon followed (Blough, 1988). Numerous reviews on fluorescent chemosensors have appeared at regular intervals to cover the research activity within this busy field (de Silva et al., 1997, 2009, Callan et al., 2005). The coupling of anthracene and pyrene fluorophores with the redox-active ferrocene emerged via 2,3-diaza-1,3-butadiene spacers (Chen-Jie et al., 2008; Martínez et al., 2006). The utility of click chemistry to redox-fluorescent sensors was demonstrated in the Ghosh lab (Thakur, 2013), who reported chromogenic and fluorogenic indicators for Pb(II) figure 3. One such molecule was synthesised from the alkyne-derived anthracene and ferrocene-azide in the presence of copper (I) iodide and 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) in N,N-dimethylformamide (DMF). The anthracene-triazole-ferrocene product is a dual mode chemosensor in aqueous acetonitrile. In the presence of lead (II) (Pb^{2+}) the solution colour changes from yellow to green, and when irradiated with 363 nm, a blue emission prevails. The Thakur team has since capitalised on azide-alkyne click chemistry to successfully demonstrate ferrocenyl-coumarin INHIBIT combinatorial logic gates (Bhatta et al., 2017; Karmakar et al., 2020). Reviews on chemosensors incorporating click-derived triazoles (Ahmed et al., 2021; Heng Lau et al., 2011) and ferrocene (Sahoo, 2020) are also available.

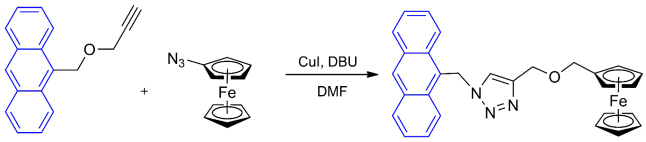

Figure 3: Synthesis of a dual-output colorimetric and fluorimetric sensor (Thakur, 2013).

During the same year of 2013, our research group was also designing a molecule with an anthracene fluorescent reporter and a redox-active ferrocene group. We coined our invention a "Pourbaix sensor" (Farrugia et al., 2013; Magri, 2009). It too works on the concept of orthogonality and uses fluorescence as a means of communication between molecules and human researchers. Orthogonality is used in the context to describe how one photoactivated process occurs independently of another process. Our objective was to design molecules that could detect for two fundamental physicochemical parameters, the acidity and the oxidisability of a solution, and we wanted to detect for both parameters simultaneously. From a logic perspective, this satisfies an AND logic algorithm (de Silva, 2013, Yao, 2020). The reason for our interest in these two conditions is that corrosion of materials, like steel, occurs at condition of high acidity (low pH < 4) and high oxidative conditions near the surface of the material. The stand-

ard industrial method for corrosion detection is the liquid penetrant test using a fluorescent dye and a UV lamp. In logic terms, the fluorescent dye functions as an elementary PASS 1 logic gate. During the corrosion process of steel, iron (III) ions are liberated, which is an oxidant with ferrocene. To detect for the occurrence of these two conditions, we designed a molecule with a fluorophore reporter, blue-emitting anthracene figure 4 and a tertiary amine for detecting protons, and ferrocene for detecting an oxidant, such as ferric Fe^{3+} ion. We employed a reductive amination reaction to bring two components together to form a C-N bond.

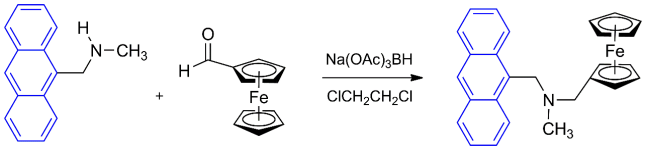

Figure 4: The synthesis of a Pourbaix sensor from an anthracene-containing molecule with an amine group reacting with a ferrocenecarboxaldehyde using sodium triacetoxyborohydride ($Na(OAc)_3BH$).

The molecule functions based on the principles of photoinduced electron transfer (de Silva et al., 2009; Spiteri et al., 2015) Initially, the molecule is not fluorescent. To activate a fluorescence response, the molecule must be irradiated with light, specifically 365 nm light from a UV lamp. However, light activation is not enough to cause the molecule to fluorescence. Within the molecule there are two photoreactions that can occur once the molecule is in the excited state. An electron can pass from the tertiary amine to the excited anthracene fluorophore, or an electron can pass from the ferrocene to the excited anthracene fluorophore. Either photoinduced electron transfer (PET) reaction prevents the sensor molecule from emitting fluorescence. The fluorophore is insulated from the amine proton receptor and ferrocene redox donor by methylene carbon spacers. An enhanced bright fluorescent signal is only observed when the sensor molecule is protonated at the amine and the ferrocene group is oxidised, having met the conditions of high acidity and high oxidisability. Figure 5 illustrates the four states of the molecule and the fluorescence output in each case exemplified as an AND logic gate.

3 Application of Pourbaix Sensors for Corrosion Detection

The cost of corrosion to the world economy is a staggering amount in excess of $2.5 USD trillion per year. Corrosion is initially invisible to the naked eye, but once it is visible, structural damage has already occurred. So what if we could detect the corrosion at an early stage

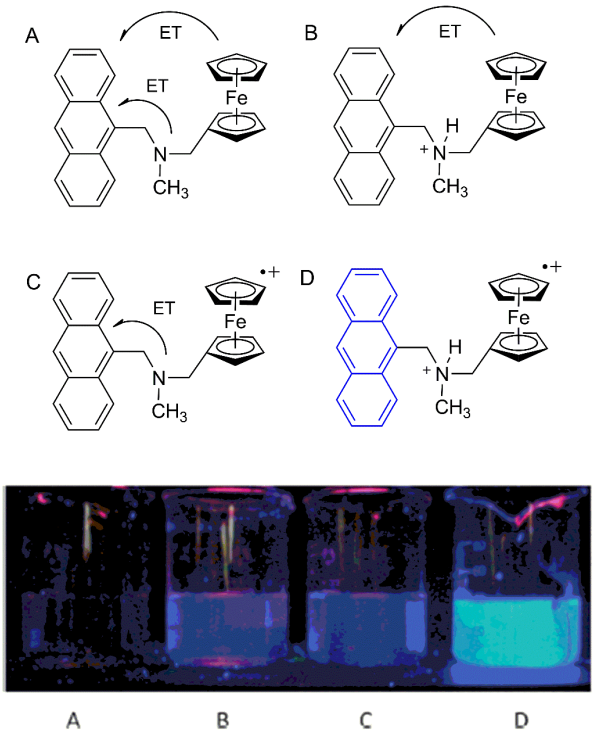

Figure 5: Top: The four states of the Pourbaix sensor after activation with light. Bottom: The fluorescent output of the sensor in methanol irradiated with a UV lamp at 365 nm in a dark cabinet. The labels A-D correspond to the four states of the molecule. Beaker D is the 'on' state. Reproduced with permission of the Royal Society of Chemistry and the Centre National de la Recherche Scientifique (Farrugia & Magri, 2013).

before it can be visually observed? Our next generation of Pourbaix sensors were designed to be brighter and and to emit at a longer wavelength with a green fluorescence (Spiteri et al., 2015, 2018; Johnson et al., 2016). Two fundamental changes were made to our next prototypes, which both contributed to increasing the fluorescence brightness. First, we changed the fluorophore to 4-aminonaphthalimide, which has photoinduced charge transfer character (Valeur & Leray, 2000). Second, we placed the fluorophore in the middle of the device so that the ferrocene and amine groups were situated at the extremities to shorten the distance for the PET reactions from ferrocene figure 4. We have succeeded with the incorporation of smart molecules in hydrogels (Scerri et al., 2022) and on polystyrene beads (Vella Refalo et al., 2019, 2018) (figure 6).

The attachment of naphthalimide logic gates to the polystyrene beads (via formation of a peptide bond CO−NH) occurs in an efficient click-like way to yield a single product in quantitative yield.

A third generation of Pourbaix sensors were developed using the perylenediimide (PDI) fluorophore (Scerri et al.,

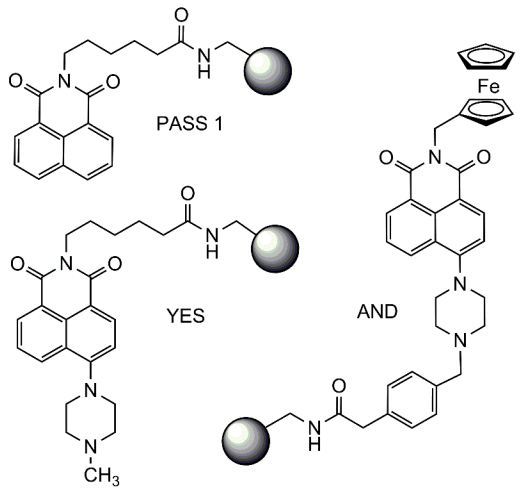

Figure 6: Examples of PASS 1, YES and AND molecular logic gates bound to polystyrene beads. The beads are not drawn to scale and have micrometer dimensions.

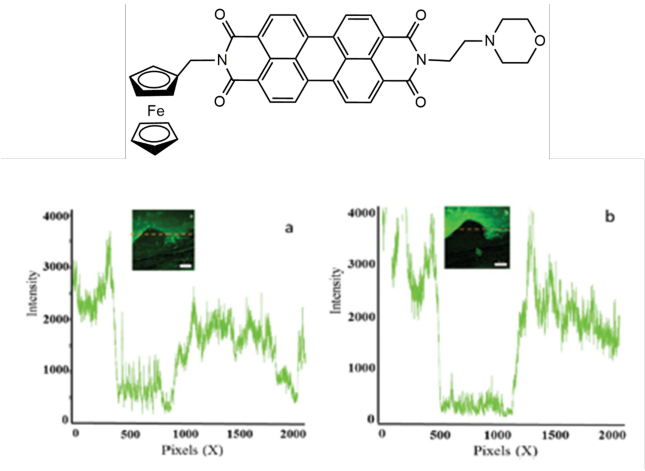

Figure 7: Confocal fluorescence microscope images (λ_{ex} = 488 nm) of a scratched edge of a polyurethane coating with a PDI Pourbaix sensor after exposure to a 0.5 M NaCl solution at (a) 0 min and (b) 90 min. Inset: The orange dotted line shows the cross section being viewed. Reproduced with permission of the Royal Society of Chemistry (Scerri et al., 2021).

2021) as shown in figure 7. PDIs are used as pigments in paints and protective coatings in the automotive industry because of their excellent thermal stability and high temperature tolerances. We incorporated a PDI molecule into a polyurethane coating on mild steel (Scerri et al., 2021). The molecule was irradiated with a 488 nm laser and observed through the eyepiece of the fluorescence microscope before and after treatment. Coated mild steel coupons were dipped into a 0.5 M NaCl solution or deionised water as a control. No discernible rust was visible to the naked eye after exposure to the 0.5 M NaCl solution after 90 minutes. However, through the eyepiece of the fluorescent microscope, the onset of corrosion was apparent. The polyurethane edge of the scratch become a bright green and a cross section image highlights a deeper 'moat' along the coating edge (figure 7). The result exemplifies the possibility of using logic-based molecules as part of the preventive maintenance protocol for vital transport such as airplanes and space vessels and infrastructure such as bridges and towers.

4 Application of Pourbaix Sensors in Living Cells

Naphthalimides are a class of molecule with proven antiproliferation activity (Tandon et al., 2017). They intercalate with topoisomerase (Topo) II enzyme and DNA to induce apoptosis by blocking the binding of the Topo enzyme to double strand DNA. We explored the possibility of using Pourbaix sensors as potential multi-targeted anticancer and fluorescent cellular imaging agents (Johnson et al., 2021). The compounds were studied *in vitro* against MCF-7 and K562 cancer cell lines. Most of the compounds did not display any cytotoxicity even after 72 hours. We therefore examined the cellular uptake and fluorescence imaging. A vibrant green emission was observed in the nucleus of the live cells within minutes, confirming the internalisation within the MCF-7 cells (figure 8).

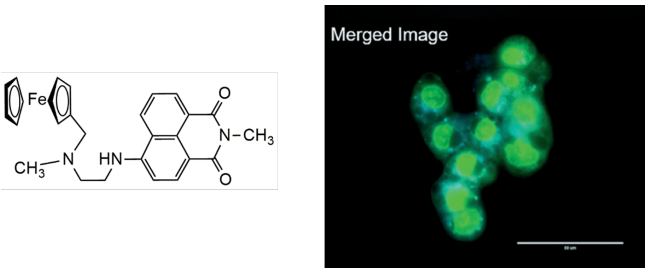

Figure 8: Fluorescent image of a Pourbaix sensor in MCF-7 cells after 24 hours incubation period. Scale bar of 50 µM. Images were captured on an EVOS FL fluorescent microscope. Reproduced with permission of the Royal Society of Chemistry (Johnson et al., 2021).

5 Application of Pourbaix Sensors for Multi-Analyte Detection

Another invention based on the concept of orthogonality and fluorescence is the concept of a 'lab-on-a-molecule' (Magri et al., 2006). Rather than detecting for two parameters, could we detect for three or more parameters including solvents, metal ions, anions, saccharides, polarity and medications (Rout et al., 2012, 2014)? It may seem trivial counting from two to three, but adding a third dimension in geometry changes the perspective from

the concept of area to volume. In medicine, detection of three targets simultaneously even better confirms a disease condition. A molecule with this enhanced capability would be a three-input AND logic gate.

For the first lab-on-a-molecule, three receptors were selected, each specific for an individual analyte. A benzo-15-crown-5 ether binds Na^+, a tertiary amine binds H^+, and a phenyliminodiacetate binds Zn^{2+}. The fluorescence reporter is the planar blue-emitting anthracene fluorophore. These four components, the three receptors and the fluorophore, are covalently attached and separated by methylene CH_2 spacers. In a solution of water with Na^+, H^+, and Zn^{2+} present at high concentration levels, the molecule glows blue. The absence of just one of these three analytes prevents the molecule from emitting a blue emission, similar to the image in figure 5.

The latest prototype of this genre is a green-emitting aminonaphthalimide lab-on-a-molecule (Scerri et al., 2019) figure 9. The sodium receptor was modified with the stronger binding N-(2-methoxyphenyl)aza-15-crown-5 ether for binding Na^+, a piperazine ring for binding H^+, and a ferrocene group for sensing Fe^{3+}. The significance of this combo of three analytes is of relevance to corrosion detection. As already discussed, high Fe^{3+} and H^+ levels are diagnostic for eroding conditions for steel objects. The presence of Na^+ accelerates the rate of corrosion.

This same trio of analytes is also of consequence to cancer. Cancerous cells have a tendency to grow much more rapidly than health cells. There is evidence that the analyte levels are often higher in cancer cells. Free Fe^{3+} is redox active and is known to contribute to various cancers including colorectal and liver cancer. The intracellular vacuoles of tumour cells are more acidic and the intracellular Na^+ levels of cancer cells can be up to three times higher than in normal tissue. Hence, the simultaneous detection of high Na^+, H^+ and Fe^{3+} could not only serve as an early warning method for corrosion of steel (Magri et al., 2014), it could also be a test for diagnosing a patient with cancer. The alternative is three separate tests and consideration of the results of the three tests by a practitioner, which will lengthen the time for the analysis. However, lab-on-a-molecule systems can perform a 'yes' or 'no' decision quickly and intelligently. It is envisioned that perhaps one day we may have a lab-on-molecule that could perform a CHEM-7 analysis, for example, for Na^+, K^+, Cl^-, HCO^-, blood urea nitrogen, creatinine and glucose from a tiny drop of blood (Scerri et al., 2019).

6 Conclusion

Chemical reactions that are fast, selective and high yielding are desirable to the chemist. Within living systems, reactions that are biorthogonal, that is, those that do not react with the complex biochemistry, are desirable to the cellular biologist for probing the cellular environment in a controlled manner. Some possible steps forward for the field of molecular logic gates is the development of molecules in more synthetically efficient ways, and the incorporation of fluorescence sensing molecules within living systems that can detect multiple analytes in an orthogonal and cooperative way. The amalgamation of the concepts of click chemistry and biorthogonal chemistry with molecular logic-based computation should yield new discoveries and inventions going forward.

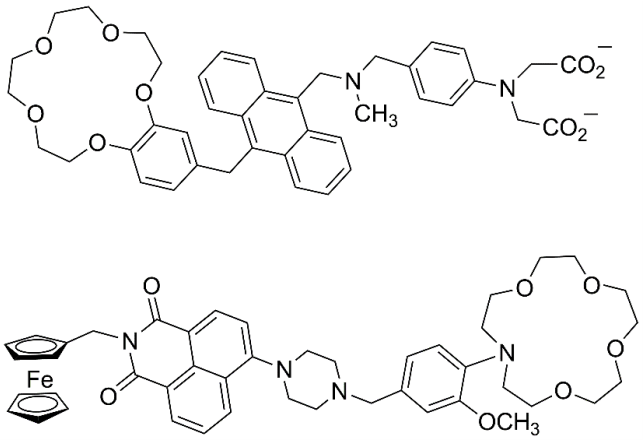

Figure 9: The molecular structures of three-input lab-on-a-molecules.

7 Conflicts of Interest

The author declares no conflicts of interests in the preparation this manuscript.

8 Acknowledgements

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Review Article

The Beautiful World of Human Haemoglobin (revisited)

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Abstract. In this article I sought to tell two stories that were closely tied to each other. One was somewhat personal. It had to do with the course of my professional life, in Malta, the USA, then back to Malta. It involved family, a handful of mentors that shaped my career and many graduate students or trainees. They combined to link with the second objective of this article to recount our contributions to Human Haemoglobinopathy studies. Our research revealed the quantitative effects of genetic co-regulators on complex phenotypes. We assumed that mutations at two to three alleles acting among two to three (globin) loci could act to express a β globin gene variant at any level between as little as 5% or as much as 100% in heterozygotes. The same type of interaction could be seen in α globin and other molecules such as KLF1, the master regulator of Erythropoiesis, and perhaps others with a similar molecular model. The new health and academic professionals that we trained, the new resources in the hospital, the new laboratories and the new International Initiatives promise well for new discoveries to promote expedited diagnoses and new treatments of Rare Disease in general.

1 Preamble

When Prof Giuseppe Di Giovanni asked me to contribute to this second volume of the special issue of XJENZA-ON-LINE to celebrate successful researchers in Malta, I thought of reviewing my research on the genetics and pathophysiology of human Haemoglobin (Hb) largely from a personal perspective. The journal first known as XJENZA was, of course, close to my heart since it was published by the Chamber soon after we had founded it in 1992 (Fig. 1) and I was President (Felice et al., 1996). Patrick Schembri had taken the initiative on the Council, and Angela Xuereb was the first editor of (Xureb, 1996). XJENZA-ON-LINE succeeded the printed version of XJENZA in 2013, with Giuseppe Di Giovanni serving as the editor for a period of 5 years (Di Giovanni, 2013). Subsequently, the role of editor was assumed by Cristiana Sebu, who currently holds the position. The initiative was supported by Peter Serracino Inglot, Mario Tabone and George Pullicino at the MCST. Marion Zammit Mangion, Robert Bort, Paul Debattista, Nicholas Gingell, Christian Scerri, and Kenneth Bartolo were the other members of the founding council.

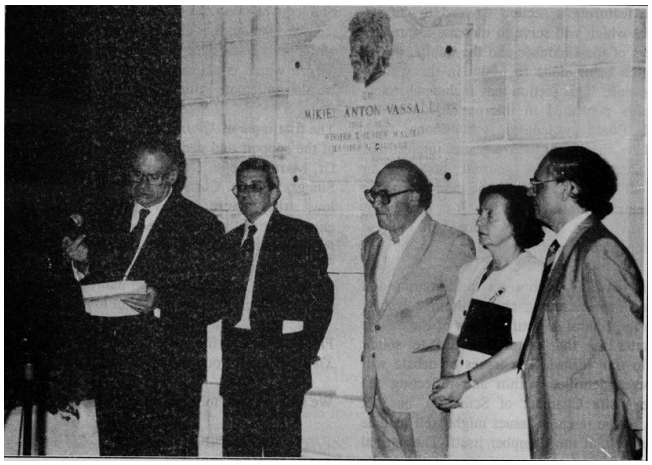

Figure 1: Inaugural event of the MALTA CHAMBER OF SCIENTISTS held on the 29 th of July 1994 on campus of the University of Malta showing left to right his excellency Dr Ugo Mifsud Bonnici then President of Malta addressing the gathering with Prof. Alex Felice, president of the Chamber, The Rev. Professor Peter Serracino Inglott, then Chairman of the Malta Council for Science and Technology and Rector of the University of Malta, Mrs Mifsud Bonnici, and Professor Victor Ferrito, President Elect.

My Story is personal as well as scientific; there was always a bit of Chemistry at home since my father was a chemist of the older tradition and he taught chemistry. Watson and Crick (1953, but see also Pray, 2008) published their manuscript on the structure of the DNA in Nature when I was a young boy starting secondary school-

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ing at the Lyceum in Hamrun. We had exceptional science teachers then. I joined the School of Medicine in 1966, and graduated M.D. in 1971; just over 50 years ago, that we celebrated with family and friends last year. Soon after reading for the Master degree in the Faculty of Medicine and Surgery, Joanna (formerly Nicolas) and I got married and left for our adventure in Augusta, Georgia, at the Medical College of Georgia in the Southeastern USA. Augusta was well located with being barely three hour drive from the State Capital, Atlanta, home of the Braves and the Falcons, the mountains of Tennessee, the old capital Savanah and the golden beaches running from Miami up North to Myrtle beach in Northern Carolina / Virginia. We lived in a mixed society, partly a cosmopolitan environment dominated by a vibrant medical academic community and the military at Fort Gordon and the typical "friendly southerners" Golf was the dominant sport. Augusta still is the Golf capital of the world with its annual Masters' tournament. In the meantime, Joanna joined the Augusta Museum as a docent and later started on her professional experience in librarianship that she later brought back to the library of the University of Malta as Deputy Librarian. Occasionally she joined the team travelling to somewhat obscure places in the forests of Southern Georgia to collect samples from highly informative families that we published. We shared the excitement and tribulations of unnerving weekends to meet deadlines and the interminable wait for the outcomes of submitted manuscripts or grant applications. Even then, though, we shared concerns about the underlying social tensions that came to the fore later. However, we remember odd pleasantries such as being introduced, the first time we went to the Augusta Symphony, as the new couple in the Huisman laboratory "from across the pond" I went through graduate school and an intensive post-doctoral training to be followed by a junior position on the Faculty at MCG then Associate Professor. We returned with a goldmine of experience, the happy memories of establishing a home away from home and bringing up a young family.

I shall give an account of my research on Hb as it developed first as a medical and graduate student at the University of Malta (Felice, 1975) and a trainee with my mentors at St Luke Hospital, later at the NIH funded Comprehensive Sickle Cell Center of the Medical College of Georgia, in Augusta, Georgia, USA (1976 - 1989; Ph.D., 1981) and then back to Malta in 1989. The late Joe Louis Grech was the clinical biochemist at St Luke's. He was specialized in Clinical Pathology with expertise in BioMedicine, mainly Biochemistry. It was the fore-runner then of what we may be calling Genomics Medicine today. He tasted the discovery of the first *Maltese* Hb variants on top of his busy clinical schedule. Joe Louis was an exceptionally fine gentleman, a superb teacher. He led by example. He once told me "young man; you cannot find what you do not look for!" In retrospect, it sounded like what today we know as a *Specific Objective* I often argued with my students that a *specific objective* could not be more than one, maybe two. He had introduced the quantification of the minor Hb component in adult blood, the HbA2 by paper electrophoresis for the diagnosis of the common thalassemia heterozygote, or trait as it is commonly known. Later, he also added the procedure of cellulose acetate electrophoresis for newborn Hb testing. The late William Bannister had returned to Malta from Oxford before I started studying physiology. He had set up a new Laboratory of Protein Chemistry in the Department of Physiology and Biochemistry to research copper bound molecules that were similar in many ways to Hb. They both bound oxygen. With Maurice Cauchi they had just reported the discovery of the Hb F Malta I [or $\alpha_2 G\gamma_2$, 117(G19)His > Arg ; Cauchi et al., 1969] and Hb St Luke [or $(\alpha_1)_2$95(G2)Pro > Argβ_2; Bannister et al., 1972] variants. Willie, as we knew him, was a committed researcher with hardly any interest outside the research laboratory. Although occasionally we disagreed, he also gave me sound advice mostly about threading the treacherous paths to securing research funding in Malta. After my M.D., I researched both Hb variants for my M.Phil. (Felice, 1975, Felice, 1977) that was among the first from the Faculty of Medicine and Surgery. The other was Marie Therese Podesta' now Camilleri Podesta'. With Roger Ellul Micallef, later Rector of the UM, the late Anton Pizzuto and the "Kapillan" of the locality we used to organize collections in isolated villages across Malta and Gozo (Felice et al., 1977) We introduced the practice of signed *informed consent* before testing. In the process, though, with Manuel Agius and George Grima we set up the first Research Ethics Committes.

We never discovered very much then! Even the Hb F Malta I homozygote was elusive, despite the high heterozygote frequency (1.8%) The low proportion of the Hb St Luke variant (< 20% of total Hb) in blood of the adult heterozygotes was intriguing though.

In the course of this research, Hb F Malta I, however, turned out be a most useful biomarker of human γ globin gene expression. It shed light on the structural and functional organization of the globin genome before genome sequencing was widely available. It was found to be uniquely in tight linkage disequilibrium with the β globin variant known as *Hb* Valletta [or $\alpha_2\,\beta_2$87(F3)Thr > Pro ; Felice et al., 1990, Kutlar et al., 1991] Whoever inherited the Hb F Malta I variant also inherited the Hb Valletta

variant on the same chromosome, in cis. With Alex Camilleri in the course of his M.Sc. (Camilleri, 2018) we found very few instances when one occurred without the other. The recombination rate in this part of the genome must have been very small. This genetic arrangement is still rather unique in human genomics. In the case of the Hb St Luke, the molecular pathophysiology gave us insight into the biochemistry of post-translational assembly of heteropolymers and models of gene expression in Thalassaemia and in Sickle cell Disease (Felice et al., 1977) and later about transcription factors (TF) such as KLF1 (J. Borg, 2010, L. Grech, 2018)

My induction into the Beautiful World of Haemoglobin at the University of Malta was followed by a longish stint on the Faculty, in the Departments of Cell and Molecular Biology and Paediatrics (Paediatric Haematology) of the Medical College of Georgia (MCG) in the School of Medicine, the School of Graduate Studies, and the NIH funded Comprehensive Sickle Cell Center in Augusta. In the process, I inherited the Huisman Laboratory to direct the Haemoglobin Research Program in the Medical Research Service of the adjoining Veterans' Administration Medical Center in Augusta. The group was globally competitive in Hb research. We were deeply engaged with challenging questions about haemoglobin, the structure, the function, the genetics and the patho-physiology. I read for my Ph.D. degree under the tutorship of Titus Huisman, an inspirational man. Together with David Weatherall at Oxford, UK and Walter Schroeder at Caltech, California, USA he was a world leader on Foetal Hb, Sickle cell Disease, and Thalassaemia (Felice, 1986) He trained me in the subtle skills of research project management starting with writing successful competitive grant applications for research funding. Actually, he tore to shreds my first effort at writing a research manuscript for publication telling me "this is philosophy, now go back home and write science" They were among other "pearls of wisdom" from my mentors that later I tried to pass on to my students.

We started with haematology and protein chemistry at the UM and evolved into molecular biology and human genomics at MCG applying a variety of analytical and experimental tools that collectively are nowadays known as "omics" within the larger scope of BioMedicine. Like stamp collectors, we made catalogues and albums of samples that later we turned into biobanks. In the process, we learnt how to "translate" biomedicine between the clinic and the research laboratory. At first, after much correspondence and missed visa deadlines, once in Augusta, I was asked to pursue the initial observation that we had made in the Bannister laboratory about the low and variable expression of the α globin variant Hb St Luke among the heterozygotes from Malta. The issue bore on the genetics of the α globin or α thalassaemia and possible effects on the biosynthesis of HbF in Sickle Cell Disease (SCD) and β thalasaemia as I shall explain below. We thought that understanding the control of globin gene expression and Hb F levels could lead to new treatment of both Thalassaemia and Sickle Cell Disease by turning the perinatal globin switch back in adults who could survive, disease free with HbF instead of HbA or HbS in blood. The data gave insight into competitive post-translational assembly of hetero-dimers between wild type and mutant polypeptides, how this accounted for the quantitative variability of certain Hb variants in heterozygotes and homozygotes, and the effect on Hb F levels in Thalassaemia and Sickle Cell Disease.

On returning to the UM and St Luke Hospital I met up again with Joe Louis Grech and William Bannister. Maurice Cauchi had established himself in Melbourne, Australia though he returned briefly (1992-2003; Cauchi, 2019) We founded a joint project between the Department of Health, then under the direction of Alfred Grech, Chief Government Medical Officer and the University while Edwin Borg Costanzi, a mathematician, was Rector. Alfred Grech secured the support of the World Health Organization for a grant to set up a specialized laboratory for Thalassaemia Testing that was further supported by the University for Hb Research (Felice et al., 1990, Buhagiar et al., 1997) George Hyzler, then John Rizzo Naudi were in the Ministry of Health. Frederick Fenech was Dean of Medicine and Peter Serracino Inglott, a philosopher with extensive interests had succeeded Borg Costanzi as Rector. On campus, we set up joint laboratories for Thalassaemia Testing and Hb Research and a brand new Laboratory of Molecular Genetics. The latter became an advanced diagnostic service for genetics in Malta under the direction of Christian Scerri. Christian was one of the first group of three doctoral graduates who specialized in Pathology and Diagnostic Molecular Genetics. We launched the teaching and applications of modern Molecular Biology in the field of human genomics and genetics medicine. We intended to build on the long tradition that the (Royal) University of Malta had in the field of human haemoglobinipathy. Frank Vella, now retired in Saskatchewan, Canada, where he had been Professor of Biochemistry had pioneered the discovery of new Hb variants in the Far East. Vella was president of the International Union of Biochemistry. The Paediatricians, Manwel Cachia and Tommy Agius Ferrante, their successor Paul Vassalo Agius had considerable experience with Thalassaemia in the clinic. I had many recollections as a trainee in paediatrics. Joe Louis had once told me (if you took my word) that the samples from the Maltese patients of the time were among those that Ceppellini in Milan had

studied to determine the levels of the minor HbA_2 to be pathognomonic of the thalassaemia heterozygote (trait). He was using a cumbersome starch block electrophoresis. Today the test is automated on a High Performance Liquid Chromatograph (HPLC) The test permitted quantification of the reproductive risk among couples to bear homozygote children sick with Thalassaemia Major. In the hospital, first St Luke, then Mater Dei, we set up a specialized Thalassaemia Clinic to serve for clinical excellence in the setting of an academic project. In particular, we sought to document the natural history of Thalassaemia homozygotes that differed in severity of the disease due to the different β globin mutations as genotyped, the Hb F levels, and possibly other "genetic modifiers" We thought that the clinical genomics might suggest alternative therapies with innovative inducers of post-natal HbF (HFIs) or erythropoiesis stimulating agents (ESAs) It continues to thrive under the direction of Christian Scerri and with the recent addition of genetics counselors known as genomic health co-ordinators.

Although the story is personal, it is also that of many mentors, teachers and later younger collaborators who trained with us and joined the new Thalasaemia Project. Soon, Monica Pizzuto, Ruth Galdies and Wilma Cassar joined in the laboratory. Ray Parascandalo, Christian Scerri, Simone Buhagiar and later Dragana Josifova and Mary Rose Caruana joined in the clinic. Ray and Christian later became consultants at St Luke/Mater Dei and Dragana at Guy's Hospital in London. We pioneered a graduate program. Christian, Mohamed Marwan and Connie Bezzina were the first Ph.Ds from the Faculty of Medicine and Surgery ever (C. Scerri, 1998, Bezzina Wettinger et al., 1999, and M. Marwan, 1998). Christian is now Professor and Consultant in Genetics at Mater Dei. Connie, (Bezzina van Imp) now occupies the Chair of Molecular Cardiology at the Amsterdam Medical Center and is elected member of the Royal Academy of Science of the Netherlands. Others followed; Godfrey Grech, Stephanie Bezzina Wettinger, Svetlana Schembri Wismayer (formerly Pulis), Marion Zammit Mangion, Renald Blundel, Steve Bonello, Rosienne Farrugia, Isabel Borg, Joseph Borg, Charmaine Vella, Alex Cammilleri, Seham ElJali and Aisha Benzetoon read for Master degrees and contributed comprehensively to our understanding of Hb Genetics and clinical patho-physiology. The doctoral graduates who worked on a variety of related questions included, Stephanie Bezzina Wettinger, Steve Bonello, Clint Mizzi, (the late) Ali Ashthar, Nikolai Pace, Joseph Borg, Laura Grech, Joanna Vella and Seham el Jali. Most now hold academic or professional positions at the University or Mater Dei Hospital and even elsewhere.

The Thalassaemia Project of Malta was approved and underwritten with a grant from the World Health Organization. We were further supported with the first Maltese EU-funded research award through the new Avicenne Program that Ugo Mifsud Bonnici then Minister of Education had secured in the preparatory phase of EU accession John Rizzo Naudi, who pioneered rare (blood) disease medicine in Malta had succeeded George Hyzler at the Ministry of Health and sustained the project. The Thalassaemia Project developed well with a strong reputation of quality care in a research setting. Furthermore, it led to the development of a newborn testing service with the later addition of hypothyroid testing, the diagnostic molecular genetics laboratory, now under the direction of Christian Scerri and an innovative driver of biobanking even at European level. With Dorita Galea and others from Eurordis, the European network of rare disease patient support groups, we co-founded Eurobiobank, the first pan-European Biobank specialized in Rare Disease Biobank-

Figure 2: Photos of personages from the past and the present who led the development of research on Haemoglobin and Thalassaemia at the University of Malta showing; a. Professor Frank Vella, who pioneered Thalassaemia testing in Malta and discovered some of the first Hb variants in South East Asia. b. Professor Joe. Louis Grech who directed the Laboratory of Clinical Pathology at St Luke Hospital where he set up Newborn testing for Human Hemoglobinopathies and inspire the further development of the research program. c. Professor Maurice Cauchi reported the discovery of the Hb F Malta I variant and later pursued his career in academic Pathology at Monash University in Melbourne, Australia. d. Professor Janet Rowely (London) Professor WH Bannister and Professor Felice at the signing of the agreement with the World Health Organization to set up the Thalassaemia Project jointly between the Malta Department of Health and the University of Malta. e. Professor Christian A. Scerri now director of the Laboratory of Molecular Genetics. f. Recent graduates and collaborators at the Thalassaemia testing and Haemoglobin research laboratory on campus showing Professor Joseph Borg, Dr. Laura Grech and Ms R Galdies.

ing (Mora et al., 2015) that I currently Chair, to be followed by the European Research Infra-Structure known as BBMRI-ERIC (see https://www.bbmri-eric.eu/) Nikolai Pace is now National Node Director, and Joanna Vella, is a manager. In Malta, we supported the National Alliance for Rare Disease Support. Undoubtedly, the number of graduates and trainees, in particular, those that went on further to develop their own projects and the number of publications were the most satisfactory harvest of the project that endured so long.

Our single over-riding research objective was to understand the mechanisms that regulated the genetic switching of Hb F to Hb A (or γ to β globin gene switching) that occurred physiologically around the time of birth. It is a critical question in human physiology. It could be described as the "holy grail" of Hb research on the assumption that, if one understood the mechanisms of globin gene switching around birth, one could then develop treatments to suppress or revert the switch in order to treat the β(/HbA) haemoglobinopathies, both thalassaemia and SCD (Olivieri et al., 1998, Orkin, 1995). Together with our collaborators in Augusta, Oxford and Rotterdam, we followed two lines of investigation according to the "Augusta Model" In the first instance, as said, like stamp collectors, by exploring the quantitative epidemiology and phenotypes of Hb variants and haemoglobinopathies among families and populations we could infer potential genetic mechanisms. In the second stage, we could put to test laboratory. In the long run, the approach proved successful with the recent the inferred mechanisms using advanced "omics" technologies in the experimental uncovering of the KLF1 locus (Bieker, 2020) as possibly the master regulator of globin gene switching and the biosynthesis of Hb F that may now lead to new treatments (J. Borg, 2010, L. Grech, 2018, 2022) Photos of a few most closely involved in setting up the project are shown in Fig. 2.

The structural organization of the Hb molecule turned out to be deceptively rather simple (see Weatherall et al., 2008 for reviews). It evolved over 450 million years ago since the vertebrate Hb of red blood cells (erythrocytes) and the Myoglobin of the musculature diverged (Vinogradov et al., 2007). It appears that although the evolutionary pressure was primarily to accommodate the requirements for oxygen in the deeper tissues of larger vertebrates, while buffering the accompanying ionic fluxes, the temperature control required by intensive oxidative metabolism may also have contributed selective pressures. The developing foetus and the brain had specific needs. Likely, both differed during development. Conceivably, foetal and adult Hb phenotypes evolved subsequently in synchrony with complex developmental control of gene switching and erythropoiesis (Peschle et al., 1985) Although commonly known as a tetramer of globin sub-units, in my opinion, Hb was better described as a duplex of two heterodimers each made up of one α and one non-α globin sub-units each bound to a heme prosthetic group that in turn bound oxygen. Each $\alpha\beta$ heteroduplex included one α-like and one non-α, or β-like globin, encoded by genes in the HBA(α) and HBG(γ) or HBB(β) loci, respectively. Various Hb molecules were resolved and quantified by physicochemical methods of electrophoresis / chromatography. Developmental regulation of globin genes resulted in the expression of stage specific Hb molecules that accounted for the chemical heterogeneity of Hb lysates from blood samples (Some were acquired, such the HbA1c due to persistent hyperglycemia of diabetes, but the most significant were dependent on developmental control of globin gene expression) (Fig. 3)

Jonxis had observed that unlike that of the adult, a substantial amount of the Hb of the newborn resisted denaturation in alkali (Jonxis et al., 1956) He referred to this fraction as "Foetal Hb" or HbF. Later, Walter Schroeder at Caltech, while sequencing the erythrocyte enzyme Carbonic Anhydrase needed a reference control. He asked for HbF globin from the Huisman Laboratory in Augusta. That actually complicated Schroeder's life because amino-acid 136 of the γ globin gave non-unitary values, as if the protein had been a mixture of two, one with Glycine and the other with Alanine at position 136 (Huisman et al., 1977) I was still at Bannister's lab and had started my postgraduate master's project with the objective of exploring the occurrence of the variant Hb F Malta 1, a variant of the $^{G}\gamma$ globin. Since the prevalence was, uniquely high (1.8%) one of my goals was to find the elusive homozygote. If the assumption of non-allelic duplication of the γ globin gene was correct, then the homozygote ought to have had only the $^{G}\gamma$ variant and the $^{A}\gamma$ wild type globin as was indeed the case. As expected, the quantitative studies and subsequent gene mapping developed a consistent picture of two non-allelic γ globin genes now known as $^{G}\gamma$ and $^{A}\gamma$, on each chromosome as Scroeder and Huisman had predicted. Erythroblasts expressed four (4) γ globin genes, 2 $^{G}\gamma$ and 2 $^{A}\gamma$ Postnatally, as Hb F declined below 1% and Hb A increased, above 95%, the HbF phenotype changed from the high $^{G}\gamma$ typical of the neonate (0.7) to the high $^{A}\gamma$ of the small quantities of HbF in a sub-population of adult erythrocytes (F-erythrocytes) to be found in the adult blood. The Hb F Malta 1 variant declined postnatally faster than the $^{A}\gamma$ globin though it remained detectable (Altay et al., 1977) The Chemical heterogeneity of HbF was shown to be a global phenomenon (Huisman et al., 1977). I was privileged to write Schroeder's obituary that

was accompanied by a chromatograph displaying the separation of the original $^{G}\gamma$ and the $^{A}\gamma$ polypeptides from the same sample that Schroeder had used for amino acid analysis, this time though, on a high resolution HPLC as shown in Fig. 3 (Felice, 1986).

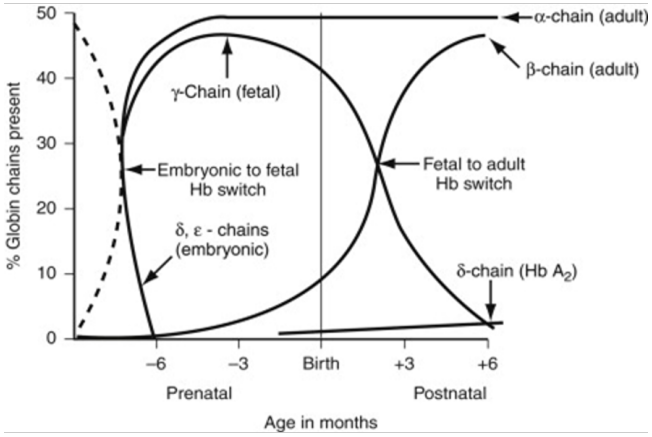

Figure 3: Graphic showing the changes of foetal to adult globin gene expression in the course of human development. It can be seen that two switching events define the transitions in globin / Hb phenotypes. The first occurred in the early embryo from ε and ζ to γ and α. The second occurred around the time of birth from the foetal $G\gamma$ and $A\gamma$ to the adult δ and β globins.

A common polymorphism of the $^{A}\gamma$ globin known as $^{A}\gamma^{T}$ or HbF Sardegna (or $\alpha_2 A\gamma_2$, 75(E19)Ile > Thr) with a frequency of around 15% in Malta and the rest of Southern Europe was also found (Marinucci et al., 1979). It was another useful quantitative biomarker in Thalassaemia research. We observed a few compound/double heterozygotes in whom each β and γ globin gene expressed throughout foetal and adulthood was biochemically marked. The quantitative Hb phenotype served to quantify in vivo the expression of each one of the globin genes during development. As far as I know, this is unique for any locus in the human genome. Residual amounts of Hb F continued to be synthesized throughout adult life and expressed within F-erythrocytes (Lennora et al., 1996). The relative distribution of the HbF among the adult F-erythrocytes varied. Jeanesse Scerri, in her Master project, suggested that it may be under control of the FLVCR1 locus that regulated the cellular distribution of the haeme prosthetic molecule (J. Scerri, 2014, Scerri J., et al., 2022)

Globin Gene Switching was accompanied by partial repression of α globin gene expression and many changes in the structure and metabolism of the erythrocytes that must be tightly maneuvered in stem/progenitor cells (Felice et al., 1979a) However, it seemed that globin gene control could be targeted separately from the differentiation of the erythrocytes for a specific therapeutic benefit.

Like that of many vertebrates, the Hb of human blood was said to be heterogeneous because the phenotype was quantitatively and qualitatively pleiotropic. Seven (7) normal haemoglobin types were physiologically expressed in the course of human development (Fig. 3) They were the embryonic haemoglobins, Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Portland ($\zeta_2\gamma_2$), and Hb Gower 2 ($\alpha_2\varepsilon_2$), up to 12 weeks of embryonic development, the foetal haemoglobins HbF ($\alpha_2{}^{G}\gamma\gamma_2$) and ($\alpha_2{}^{A}\gamma_2$) up to around the time of birth that was taken over by the adult Hb A ($\alpha_2\beta_2$) with a minor HbA2 ($\alpha_2\delta_2$) making up less than 3.5% in the absence of a β thalassaemia. The heterogeneity reflected the patterns of expression of the α-globin gene locus on human chromosome 16, and, the β-globin gene locus on human chromosome 11 (Fig. 4) Comprehensive reviews can also be found in Weatherall et al., 2008 and an extensive bibliography onthis website.

The α-globin gene locus (Fig. 4) covered a region of around 30kb on the short arm of chromosome 16, contained the embryonic ζ_2 gene, three pseudo genes, $\psi\zeta 1$, $\psi\alpha 2$ and $\psi\alpha 1$ and the two-α globin genes - $\alpha 2$ and $\alpha 1$. Their regulation is extensively described in Higgs et al., 2008. We quantified repression of $\alpha 2$ and $\alpha 1$ accompanying the $\gamma \rightarrow \beta$ transition (Felice A., Huisman THJ., 1997) The non-α or β-globin locus (Fig. 4) was found on the short arm of chromosome 11p15.5 and spanned a region of around 90Kb as described in Fig. 4. The cluster 5' to 3' comprised in this order; the embryonic ε-gene, two foetal genes, G_γ and A_γ a pseudo gene $\psi\beta$, and the adult δ and β genes. Apparently, the nearly balanced biosynthesis of the α and the γ or β globins was autonomous and a deficiency of one led to an excess of inflammatory globin precipitated in the form of haemochromes and free haeme in the erythroblastic islands of the bone marrow (Romano et al., 2022).

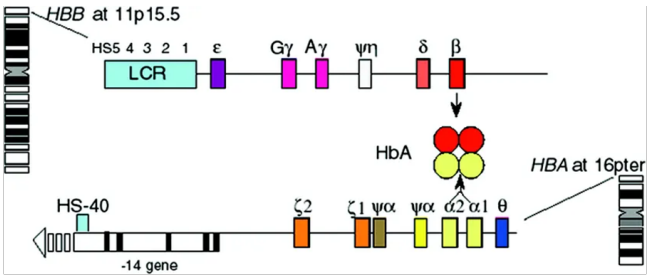

Figure 4: Structural organization of the globin genes. The duplicated α (i.e. α 1 and α 2) were mapped to p16 flanked by an embryonic ζ and an unexpressed τ genes spanning 150Kb. The non-α loci were mapped to p11 in the same order in which they were developmentally expressed i.e. ε, $G\gamma$, $A\gamma$, δ and β. TF binding sites are included among these sequences including for KLF1. A Locus Control Region can be found at the 5' end.

Family studies revealed at least four loci that con-

troled Hb F levels in adults known as co-regulators: HBB (11p15.4/ the XMN1 site; Gilman and Huisman, 1986), HBS1L-MYB (6q23.3; Close et al., 1994) and the BCL11A (2p16.1; Thein et al., 2007) and KLF1 (J. Borg, 2010). Indeed, early clues (Huisman et al., 1975) had inferred important DNA regulatory sequences between the foetal $^{A}\gamma$ globin genes and the adult β globin genes. A poly-pyrimidine rich sequence located here attracted numerous protein complexes that together formed a repressor-like complex. It looped back on itself to silence the foetal gamma globin genes in adulthood (Bank, 2006). Other co-regulators should be discoverable. This may be represented by the "KLF1 Interactome" in our model.

Globin gene expression was controlled alongside the control of Erythropoiesis (Peschle et al., 1985) Two cellular pools governed the definitive, foetal and adult lineage of haematopoiesis. One consisted of a hierarchy of stem and progenitor cells during which a genetic program of lineage specific cellular differentiation was assembled by tightly controlled gene switching events. KLF1 with its very strong promoters had an open configuration and very active at this stage (Heshusius et al., 2022, Herseus et al 2022) It was succeeded by a later terminal phase in bone marrow during which the pro-erythroblasts expressed the determined program of gene expression to differentiate into circulating reticulocytes and erythrocytes. The resting immature progenitors, the BFUe and the CFUe, either spontaneously entered a pathway leading to cell death (apoptosis) or were rescued by Erythropoietin to enter the pathway of erythropoiesis. The KLF1 gene had a closed configuration and inactive beyond this stage (Heshusius et al., 2022) though the protein persisted (Nuez et al., 1995) It involved other TF(s) such as MYB, Tal1, Lmo2, and GATA1 (reviewed in Grech L. 2022) and likely a few others that might assemble into the "KLF1 Interactome" and that we are pursuing in our current research.

The chemical heterogeneity in red cell lysates was the result of developmental changes during foetal and adult development and seen worldwide. Population studies yielded a comprehensive picture of global Hb epidemiology that gave insight into biochemical mechanisms of post-translational protein assembly by interplay between mutations that impaired protein structure and function as in many α and β globin variants including the Hb S of SCD (Perutz et al., 1968). They were commonly due to single point mutations in the exons of the globin genes, or, of genetic regulation as in thalassemia or the Hereditary Persistence of Foetal Hb (HPFH) or both that were associated with quantitative repression of globin gene expression. The most common variants seen among the Maltese are shown by electrophoretic identification and chromatographic (HPLC) quantification (Fig. 5)

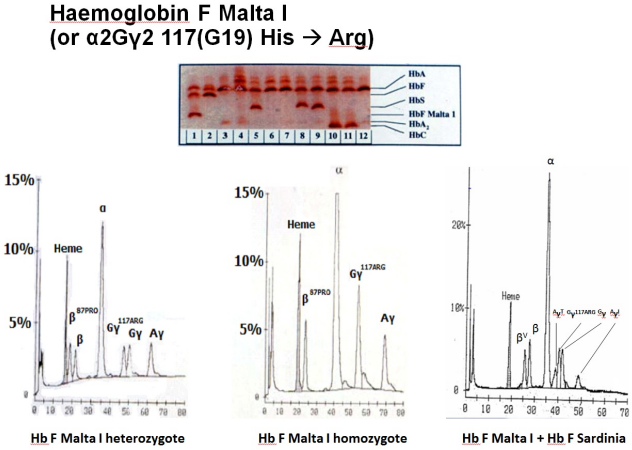

Figure 5: Chemical heterogeneity of Hb phenotypes revealed by iso-electric focusing (top panel) and reverse phase HPLC (bottom panel) of selected Hb variants commonly see among the Maltese. It can be seen that the globin products of the six globin genes active in foetal to adult switching could be uniquely resolved and quantified among Hb F Malta I heterozygotes with or without Hb F Sardegna and the rare Hb F Malta I homozygote.

Two observations were made. The first was that the proportion of β globin variants such as Hb S, Hb C, Hb E, and Hb Leslie among heterozygotes could vary from as little as around 5% to the 50% anticipated (Huisman et al., 1977, Felice et al., 1982, Felice et al., 1978, Felice et al., 1981a, Felice et al., 1981b, Steinberg et al., 1986) Given that they were mutations on one of the two β globin genes on either one of the two parental chromosomes, in trans, one would normally have expected levels of the Hb variants at about 50% of total Hb in the heterozygotes (with one of the two mutated e.g. $[\beta^S/(\beta^A + \beta^S)] = \pm 50\%$) The lower values were accompanied by a corresponding degree of microcytosis (MCV $<$ 80Fl) similar to a thalassaemia, if iron deficiency had been excluded (Steinberg et al., 1986, Felice, 1986) The second observation was that unlike the β globin variants, the α globin variants in association with a microcytosis were associated with higher levels of the α globin variant as in the case of Hb G Philadelphia (Felice et al., 1981b) At that time, I was following up on the discovery of the α globin variant Hb St. Luke that had been reported earlier by Bannister, Grech and their collaborators in Malta and Augusta (Bannister et al., 1972) Like four other variants, Hb Denmark Hill (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Ala}\beta_2$; Wiltshire et al., 1972) Hb G Georgia (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Thr}\beta_2$; Huisman T. H. J. et al 1970) Hb Rampa (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Ser}\beta_2$; de Jong et al., 1971) and Hb Godavari, (or $\alpha_2 95(G2)\text{Pro} \rightarrow \text{Thr}\beta_2$; Wajcman et al., 1998) Hb St Luke resulted from

the amino acid replacement at the α95 position of the α globin. It occurred at the $\alpha_1\beta_1$ interface that held the unlike globins tightly in the heteroduplex with little movement during oxygenation/de-oxygenation cycles. It was less flexible than the symmetrical $\alpha_1\beta_1$ interface and a stronger bond. The five mutations at the same position weakened the $\alpha_1\beta_1$ interface. They resulted in very low levels in the heterozygotes ($5-10\%$) compared to the 25% expected on the basis of a genome with 4 α globin genes active in the erythroblast ($\alpha^X/[\alpha^X\alpha/\alpha\alpha] = 1/4$ or $\pm 25\%$)

When I joined Huisman's group in Augusta, I was given a small laboratory that I modified to explore globin biosynthesis with radio-labeled amino acid precursors and a chromatographic method commonly known as the Clegg columns. I had micro-miniaturized the procedure to ask the question whether some form of thalassaemia was acting, possibly at the post-translational level to account for these observations. If so, the next question was how would the same happen in homozygotes, in particular, the β Thalassaemia Homozygotes with Thalassaemia Major, and, the Hb S homozygotes with Sickle Cell Disease. These experiments are extremely difficult to conduct today because it is nearly impossible to procure radioactively labelled amino acid precursors. Instead, Elaine Fenech is seeking to quantify gene expression with contemporary functional genomics to quantify globin gene expression with mRNA ratios as part of her Master's project.

Several families from Malta and rural Georgia joined our studies by sharing samples and data. We worked with Institutional Review Boards as Research Ethics Committees that were arising following the experience with kidney transplantation and the Helsinki declaration. On reflection, much progress could be done by working closely with patients and families in the community assuming a "unitary value" of health data without the implications of "secondary data" as now entrenched in the GDPR. I thought it needed considerable revision by patients, families and their personal health professionals. The results, partly, summarized in Fig. 6 showed that the proportional level of those β globin variants, with positively charged mutations at the $\alpha_1\beta_1$ interface, was decreased by co-inheritance of an α thalassaemia. The diminished competitivity of the variant β^X globin compared to the wildtype β^A globin for the diminished amounts of the α globin to assemble into $\alpha\beta^X$ or $\alpha\beta^A$ hetero-dimers led to the assembly of fewer heterodimers of the β^X variant compared to the wildtype HbA. In contrast, a β thalassaemia (+) mutation in trans raised the relative quantities of the β^X variant by diminishing the production of the β^A globin in cis. In fact, quantitative and molecular studies such as these allowed us to quantify the in vivo effects of any β^+ thalassaemia mutation (M. Marwan et al., 1999, see also HbVar, Hardison et al., 2002). We saw this in the case of the Hb Valletta variant among the Maltese (C. A. Scerri et al., 1993, †Felice A et al., 2016) Counter-intuitively, a concurrent α thalassaemia raised the proportions of the α globin variant while a β thalassaemia decreased it. Gene mapping and re-sequencing confirmed the conclusion reached with biosynthesis (Felice et al., 1981a, Felice et al., 1981b, Felice et al., 1982).

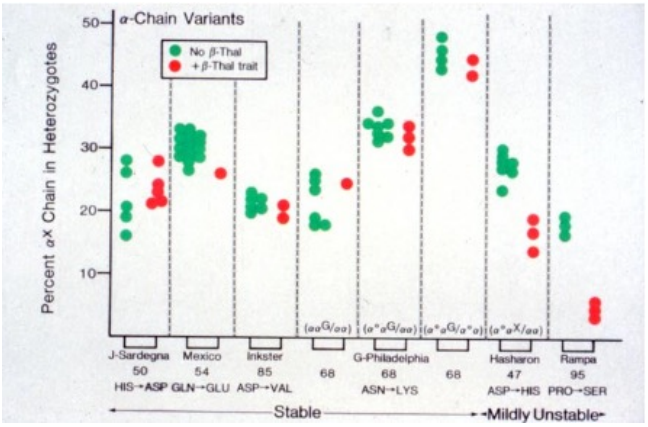

Figure 6: Quantitative epidemiology of selected α globin variants with different mutations in the α or β globins among heterozygotes from Malta and elsewhere with an associated α or β thalassaemia.

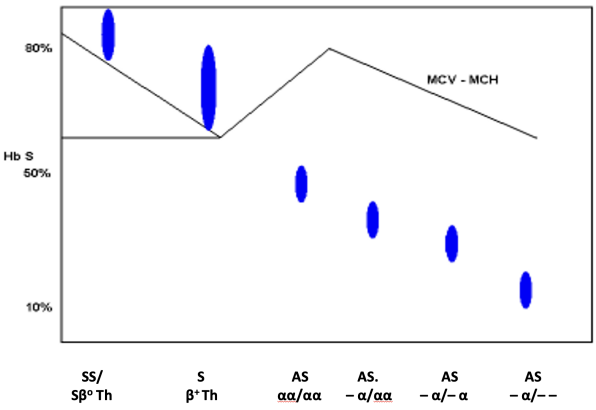

Figure 7: Quantitative epidemiology of selected β globin variants with different mutations in the α or β globins among heterozygotes from Malta and elsewhere with an associated α or β thalassaemia. The data suggested a generalized model by which the level of any heteropolymer with a structure resembling that of Hb could be decreased or increased by interplay between dys-regulatory and dysfunctional mutations affecting competition between pairs of sub-units at the level of post-translational assembly.

We asked whether the conclusions could be generalised to account for the biosynthesis of other proteins with a similar heterodimeric structure by interplay between dysregulatory mutations such as a thalassemia that decreased output, and dysfunctional mutations that decreased stability or assembly of polymeric molecules. Many molecules of body defence mechanism, immunity and inflammation, fell in this category. They had a higher order structure similar to Hb and followed a model that could account for variability in expression in health / disease. Ali Ashtar (2008), Abou Hussein (2009, 2011), Nikolai Pace (2013) and Seham Eljali (1975) have sought to apply the model to Type 2 Diabetes Melitus. It was intriguing to note further that the same mechanisms could account for the variability of the Hb and erythroid phenotype in the KLF1 deficiencies that presented with a ψ thalassaemia (see below) We found KLF1 gene frameworks with mutations in the promoter sequences associated with coding sequence mutations (unpublished observations/manuscript in preparation) The diverse KLF1 frameworks were assembled into various haplotypes/genotypes that we are exploring with respect to corresponding haematological and Hb phenotypes among families with β thalassaemia (Attard, 2020, E. Fenech, 2023).

α-Thalassaemia most commonly arose from deletion(s) of one or both of the two α globin gene in cis ($\alpha\alpha/$) on chromosome 16. Thus, functional erythroblasts with diploid genomes could express any from 4 to 0 α globin genes. It was found most commonly in its milder form with one α globin gene deleted in cis (α^+ Thal or $-\alpha/$) among Africans, or in its more severe form with both α globin genes in cis deleted (α^o or $--/$) in Asians. One of the largest α globin gene deletion assocated with α^o thalassaemia was found in a Black family associated with HbS that also had Hb H disease or the $\alpha/--$ genotype (Felice AE et al 1979) Only a handful of others are known world-wide. Although α globin variants can be found, α Thalassaemia is less common in Southern Europe and the Mediterranean. In Malta, we came across α^+-thalassaemia in the differential diagnosis of microcytosis having excluded the common iron deficiencies, the β thalassaemia heterozygotes and now the new KLF1 deficiencies with a φ thalassaemia. The few significant patients in our clinic with Hb H Disease due to $-\alpha/--$ were Asians though they had relatively quite a milder clinical condition than elsewhere; maybe they ate better here!

However, as indicated above, the haematological and clinical consequences of interplay among homozygotes with Thalassaemia and SCD was significant. Clearly, the co-inheritance of an α thalassaemia had the obvious benefit of decreasing the severity of the imbalanced (α/β) globin biosynthesis in β thalassaemia. Consequently, the dyserythropoiesis due to excessive haeme and α globin was weaker. The effects of an α thalassaemia on the haematological development among the Juvenile SCD that we uncovered together with Graham Serjaent's clinic in Jamaica was more complex (Felice et al., 1987, Sargent G., et al. 1987). It revealed dependencies of the genetic interplay underpinned by the physiological and energetic requirements of early-stage growth in juveniles ($<$ 20 years old) and the insidious onset of an inflammatory siderosis. The same was observed regarding the early stage haematological and clinical sequel of even mild β thalassaemia in Maltese juveniles (Vella, 2018, Benzetoon, 2022, Felice AE et al., manuscript in preparation). Many of the developmental haematological data, when charted resembled those of allosteric enzyme kinetics, the Hb oxygen dissociation curve or the denaturation of nucleic acids maybe reflecting a "Saturation Effect" possibly associated with the bio-energetic requirements of early development and the onset of iron excess over iron deficiency of childhood (Sebu. C., and Felice AE., unpublished observations) These prospective observational studies shall be continued within new international consortia (INHERENT; see below). Pre-conditioning of the bio-energetic balance and control of the underlying inflammation may permit newer HbF inducers (HFIs) and Erythropoiesis Stimulating Agents (ESAs) to act better, particularly in thalassaemia to prevent long term complications.

The sickle haemoglobin (HbS) resulted from a mutation in the β globin gene that substituted glutamic acid, the sixth amino acid of the β globin, to a valine (Perutz et al., 1968) The mutant Hb S moleculae became insoluble and polymerized under hypoxic conditions in the erythrocyte. The rate of polymerization depended on the 40^{th} power of the (intra-cellular) HbS concentration. Repeated cycles of oxygenation and de-oxygenation with accompanying polymerization and de-polymerization of the Hb damaged the erythrocyte membrane. It expressed Phosphatadyl Serine externally and became adhesive to the vascular endothelium. The micro-capillary vasculature was liable to occlusion by adhered sickled cells, localized hypoxia and tissue infarction. HbF was a physiological inhibitor that delayed nucleation of polymerisation. Powars et al., (2005) best described the condition as a chronic haemolytic anaemia punctuated by episodes of vaso-occlusion until the natural history was "catastrophically" altered by a major event such as acute medullary aplasia, or splenic sequestration, stroke, renal failure, or even death. The severity among patients differed markedly with some being barely symptomatic with occasionally a microcytosis or higher HbF while others, albeit less anaemic, spent miserable weeks in extreme pain due to body-wide vaso-occlusive episodes. To

complicate matters, paediatricians were describing a different clinical picture among juveniles than the adult medicine physicians. Together with Graham Sargeant group in Jamaica, we surmised that the common α thalassaemia might decrease the MC(HbS)C, or favour the assembly of Hb F over HbS, or likely both, to decrease the nucleation rate and inhibit intra-erythrocytic polymerization (Stevens et al., 1986, Felice et al., 1987)

Virgil McKee and his wife Kathleen, both paediatricians, joined us from the US Army Medical Corps to take direction of the Paediatric Sickle Cell Clinic that Alex Bruce Tagoe and I had set up in Augusta. Together, we assembled a network of statewide sickle cell clinics to follow the neonates diagnosed by newborn testing and to explore objectively the natural history of SCD. A considerable biomedical data-set accompanied extensive biosynthetic and later genetic analysis served to uncover two important effects of HbF and α thalassaemia on the clinical phenotype of SCD and that could be helpful to evolve new treatments. Deeper engagement with communities in the course of biomedical research and clinical trials has continued to evolve with dynamic eConsent procedures with AI applications on Smart Phones. We have been exploring the added value of a Research Partners' Co-Operative with interested societies and look forward to put them in practice (e.g. see midata.coop).

We made two significant observations. The first was that the rate of post-natal decline of Hb F was considerably delayed in SCD compared to the children with normal Hb type (AA) and even Thalassaemia homozygotes. It reached levels below the 15% at which Hb F was no longer inhibitory of polymerization by the age of 7-10 years. That was approximately around the age that the clinical picture change from paediatric-type to adult-type began to emerge. However, at the time, we could not see an effect of the α thalassaemia on the Hb F/Hb S levels. In retrospect, re-evaluating the data from a different perspective revealed a marginal effect. The second was that, in a dose dependent manner and after HbF had declined below 15% by 7- 10 years of age, the α thalassaemia decreased the MC(HbS)C diminishing the nucleation rate of polymerization and the haemolytic rate. Consequently, although the anaemia was milder, the increased erythrocyte survival increased total Hb / HbS, increasing whole blood viscosity, decreased flow and tissue oxygenation with sporadic infarcts in various organs. Life span improved but quality of life deteriorated due to the effects of the α thalassaemia on whole blood viscosity and the endothelium.

In the meantime, following observations on experimental animal models, Hydroxyurea, well known in paediatric haematology (Dingli et al., 2006) was shown to maintain Hb F levels above the predicted 15% that had emerged from our studies and benefited the SCD children by diminishing physico-chemical nucleation and clinical consequences. The story substantiated the value of newborn testing for expedited diagnoses of rare disease in general and the discovery of new treatments subject to the availability of samples and data with a unitary purpose in Bio-Medicine. Currently, Eurordis and Euro-Biobank strongly argue along the same lines in promoting National Rare Disease Platforms that included the human haemoglobinopoathies within the terms of the relevant European Reference Networks of hospitals and clinics (e.g. Euro-BloodNet)

SCD, like Hb H Disease and Hb E Disease were new clinical challenges in the health system of Malta. Hypermigration in the Central Mediterranean changed the quantitative Hb Epidemiology of Malta and neighboring Southern European countries. The data from Hb Neonatal testing (Galdies et al., 2023) acquired over the last 35 years showed that while the national birth rate declined markedly, the HbS and other African or Eastern Hb variants such as HbE now accounted for nearly one third of all Hb variants that were encountered. A handful of patients with SCD have presented to the clinics. The same must be happening with other, less visible rare disease. They are new clinical challenges in particular in the emergency setting across Europe.

β Thalassaemia became the specific objective of my research after I returned to Malta with the Thalassaemia Project in the late 1980. β thalassaemia arose from partial or complete deficiency of β globin biosynthesis. Unlike deletional α thalassaemia, single nucleotide substitutions or other mutations in the β globin gene gave rise to most common types of β thalassaemia. The HbVar and ITHAgene databases collected details of the mutations and deletions worldwide (Giardine et al., 2007, LeDere et al., 2009, Kountouris et al., 2021) The Thalassaemia International Federation joined patients and families with physicians and experts in biomedicine from across the world. In 1976 we had hosted the International Thalassaemia Meeting that I was privileged to chair. ITHANET was an EU funded IT platform for data sharing INHERENT was a new research network of thalassaemia clinics and laboratories to promote research. These organisations and EURORDIS acted strongly at pan-EU level to promote patient values in health services and research on Rare Disease in general. The National Alliance for Rare Disease Support represented them in Malta. Human haemoglobinopathy, like the Haemophilia community, led to an inclusive model for rare disease management in partnership with patients and families. It created the worldwide context for expedited diagnosis and new treatments.

Classically, β thalasaemia Heterozygotes were not an-

aemic but displayed microcytosis and typically, an elevated Hb A2 ($>$ 3.5%) that was pathognomonic. Actually, silent thalassaemia presented with a normal Hb A2 and the new ψ thalassaemia may have borderline Hb A2 due to KLF1 deficiencies (G. Grech, 2020) The Hb F was occasionally elevated depending on the mutation and the co-regulators. The compound heterozygote with α and β thalassaemia was normocytic but still had an elevated Hb A_2. We identified the β globin mutations that accounted for the thalassaemia of Malta and quantified the allele frequency of each (C. A. Scerri et al., 1993) Collectively they amounted to a heterozygous carrier rate of around 1%. The β^+ IVS-I, 6C, a relatively mild mutation first found among patients from Portugal was the most frequent in Maltese heterozygotes (66%) The β thalassaemia heterozygote, commonly known as thalassemia trait, was physicaly healthy albeit subject to reproductive risk. Older heterozygotes with concurrent cardiometabolic or inflammatory conditions may require closer scrutiny in the context of common MTHFR and folate deficiencies that impacted inflammatory hyperhomocysteinaemia. A formal trial has yet to be reported.

β Thalassaemia homozygotes suffered a severe medullary dyserythropoiesis caused by excessive inflammatory Haeme and precipitation of the excessive α globin as haemochromes on the membrane of the erythroblasts. The excesses inflamed the medullary eco-system of erythroblastic islands assembled around a central macrophage. It resulted in a severe chronic haemolytic anaemia and, disturbed iron traffic giving rise to tissue wide siderosis and a presumed inflammation or hypercoagulability, since infancy. Complications arose in many tissues due to long term hypoxia, siderosis and the underlying inflammation. Hb F in blood lysates (mg/dl) may be elevated by increased biosynthesis or, more often, by preferential survival of the F-erythrocytes with higher HbF due to the heterocellular distribution as explained above.

The Thalassaemia Clinic provided resources to make a genetic diagnosis with counselling of the heterozygotes and couples at risk and for the long-term care of the homozygotes within guidelines of the international research partnerships. In fact, we developed a unique long term prospective clinical cohort to define the natural history of the different β globin mutations, some severe, some mild. Christian Scerri's doctoral project served to define the genetic causes of thalassaemia in Malta and made the initial clinical observation that children with even the mild mutations required treatment as intense as others with the more severe mutations through the juvenile ($<$ 20) years. Mohamed Marwan (1998) conducted similar studies on patients from Libya providing contrasts between the two that could be further compared with related data from our collaborators in S. Italy. Marwan's research further served to quantify the dysfunctional output of the β thalassaemia mutations to improve the classification of disease. Although β^+ IVS-I, 6C mutation diminished β globin output only by two thirds [MC(HbA)C = 3.1pg/RBC] compared to others that abolished it or nearly so, the clinical outcome among juveniles appeared to be the same. As in the case of SCD the severity of the β thalassaemia varied quite markedly. Most patients required life-long transfusion and chelator therapy unless successfully transplanted. Rare, very informative patients were demonstrably homozygotes but without many signs or symptoms. Whole genome sequencing on these can provide much new insight into pathogenesis. Clint Mizzi (Mizzi, 2016) ran a comparative genomic profile between the common mild β+-IVS I, 6C of Malta and the wild type genome that did not reveal additional genomic variation. Perhaps, a similar exersise with the more severe mutations such as the β^o IVS-I, 110A of Cyprus might have yielded a different result. At the genomic level, a comprehensive genotype - phenotype connection classified the severity of the β thalassaemia by virtue of four genetic / molecular parameters. They were;

1. The severity of the β globin gene mutation and the suppression of β globin biosynthesis
2. The co-inheritance of α thalassaemia to diminish the degree of α/non-α imbalance
3. The de-repression of γ globin to compensate for the β globin deficit (and the excessive Haeme)
4. The transmission of co-modifiers that could affect organ-wide complications (e.g., MTHFR$\pm$ / others) in adults

The socio-economic conditions and the quality of care had a direct influence too. Many times, they depended on the availability of national resources. Apart from stem cell and genetic therapies there shall remain a need for improved conservative management with transfusion and chelation and potential molecules that could function as ESAs or HFIs cost effectively world-wide.

In contrast, the clinical classification of β thalassaemia left much to be desired. While the older denominations into minor, intermedia or major was not sufficiently discriminatory and failed to match the genomic classification above, the newer classification reflecting transfusion dependence was subjective and lacked a clear objective, quantifiability suitable for robust Genome Wide Association Studies (GWAS) The clinical genomic prospective study served to establish an objective score calculated from the volume of erythrocyte transfusion, the pretransfusional Hb (G/dL) and BMI (Bugeja, 2008, Vella, 2018, Benzetoon, 2022) The simpler objective quantific-

ation of the early course of the disease should be better suited for GWAS and related research with ESAs and HFIs.

Like others, we had sought to explore the therapeutic value of Hydroxyurea, that had proven useful in SCD. Partly by acting on stem or progenitor cell kinetics and partly, by epigenetically re-architecturing the $\gamma-\beta$ locus, HbF increased and sickling was inhibited. A short clinical trial in Thalassaemia conducted together with students from the Department of Pharmacy (Galea, 2005, Felice et al., 2007) gave disappointing results. Hydroxyurea induced a rapid medullary hypoplasia that we assumed due to the medullary inflammation. Similar outcomes were encountered at other clinical centers. The matter of hypercoagulability and inflammation has plagued clinical thalassaemia research for many years. It has not yet been possible to pin down a secure biomarker that reflected the significant pathophysiology. Neither the comparative genomic review of Mizzi (Mizzi, 2016) nor the protein marker exploration of Vella (Vella, 2018) or the leucocyte transcriptomics of Benzetoon (2022) in association with the innovative objective clinical score have as yet yielded robust biomarkers. The question is important because it seems that ESAs and HFIs action may be obstructed by the underlying inflammation before they could be effective at the globin gene level. Possibly, if Newborn Testing identified all β Thalassaemia and Hb S (SCD) homozygotes at birth, early use of Hydroxyurea or an improved HFI and ESA could sustain high Hb F before the onset of (medullary) inflammation, to prevent long term complications in both conditions. HU may have proven more useful in SCD because the inflammation of SCD acted on the peripheral vasculature rather than the bone marrow as in thalassaemia.

In the meantime, KLF1, a known erythroid transcription factor, emerged as potentially a master regulator of Globin Gene Switching that could have served to be targeted by new ESAs/HFIs to increase HbF for the benefit of SCD and β Thalassaemia. We came across KLF1 while following a family that had been referred to our clinic because of iron resistant microcytosis. The nuclear family, now known as FamF1 had borderline microcytosis, a normal HbA_2 with high Hb F, a phenotype akin to an HPFH, with a normal β globin gene sequence. Joseph Borg took this up first as part of his MSc in the Ithanet project and later for his doctoral project. Ruth Galdies in the testing lab found 6 others with very variable Hb F that were further studied by Laura Grech for her Ph.D. and then followed up by others in the course of our longer term research.

HPFH was thought to derive from errors of globin gene control that sustained continued activity of the γ globin genes beyond the peri-natal period without any haematological impairment. However, whenever deeper studies of globin gene expression were possible, some degree of imbalanced α/non-α biosynthesis emerged; thus, the borderline between HPFH and certain types of β Thalassaemia was somewhat blurred. Furthermore, some types of HPFH expressed Hb F in all erythrocytes (homocellular) or in an expanded population of F-erythrocytes (heterocellular) Some were due to sizable deletions within the $\gamma-\beta$ locus, others were due to meaningful mutations in the $^{G}\gamma$ or the $^{A}\gamma$ promoters. The deletions revealed in cis DNA sequences involved in switching while the promoter mutations revealed TF binding sites (Fig. 4; see Weatherall et al., 2008)

Many more members of the original nuclear family (FamF1) joined the research. GWAS at Sjaak Philipsen's Laboratory in Rotterdam (Erasmus MC) by Joseph and Godfrey Grech led us to a locus on Chromosome 19 that could be responsible for the phenotype, close to the KLF1 TF locus. Sequencing identified a truncation mutation (p.Lys288Ter; rs267607202) It produced a shortened KLF1 protein missing the DNA zinc finger essential for binding to the DNA. The subjects with the higher Hb F had multiple mutations in KLF1 both in cis and in trans. Laura Grech addressed the matter by sequencing the KLF1 of a few hundred patients from our clinic and the biobank with borderline HbA_2 and undiagnosed microcytosis. She found additional mutations in KLF1, some in the promoter and some in the coding sequence and she quantified the functional capabilities of the promoters that were found to be very strong. Many families could now have a new diagnosis, known as ψ-thalassaemia, and a better estimation of risk that they lacked before. This research won us prizes at the second European Biobank Week (Milan 2000) and the biennial Haemoglobin Switching Meeting (Oxford 2018) As in the case of the chemical heterogeneity of the Hb variants that had occupied my interests earlier on, it was concluded that the levels of KLF1 in very early stem or progenitor cells were subject to interplay between rather common dys-regulatory mutations in the (powerful) promoters and dys-functional mutations in coding regions of the gene. It seemed that the KLF1 promoter activity was very strong in the stem/progenitor cellular pool and acted on many erythroid specific loci (Fig. 8) Early-stage de-repression flooded the stem/progenitor cells with KLF1 to occupy and activate promoters of a cognate panel of other loci required for further development of the erythrocyte lineage. They de-repressed the γ globin genes at first followed by the β globin genes perinatally. KLF1 itself was subsequently repressed in the pro-erythroblasts (Herseius et al., 2022). The phenotype of a ψ-thalassaemia or HPFH appeared following the cu-

mulative effects of several mutations. In fact, the highest Hb F among FamF1 was seen in a double heterozygote that depressed the promoter function considerably. It should be possible to mimic it pharmacologically to increase the Hb F of patients with minimal side effects on erythropoiesis and no known parallel effects elsewhere. A broad drug screen and a wider "omics" profiling among additional families with diverse KLF1 genotypes could be useful.

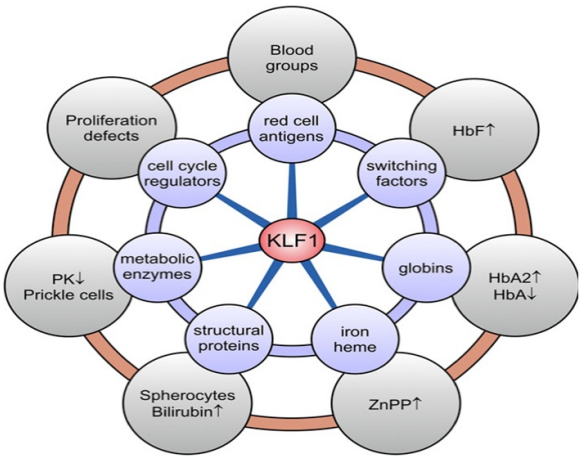

Figure 8: The KLF1 transcription factor now known as master regulator of erythropoiesis and that exhibited remarkable pleiotropy in the pathophysiology of the erythrocyte.

Although considerable headway has been made with cellular and gene therapies, a persistent need for a small molecule therapeutic remained for many patients world-wide. The severity of disease varied and the suitability of clinical settings varied just as much. The ongoing research on physiological mechanisms of globin gene control along the lines reviewed above drives the continued search for a safe and effective one.

As others take over, the way forward is to strengthen what has worked, to embed newborn testing and biobanking for expedited diagnosis and the discovery of new treatments, to look for more effective models, to innovate and improve among the European partnerships with Euro-BioBank/BBMRI-ERIC, the TIF, INHERENT and possibly others.

On reflection, we have uncovered rules governing the assembly of higher order molecules that possibly based on the KLF1 transcription factor regulated the developmental switching of human foetal to adult Hb phenotypes. Further definition of these biochemical and genetic mechanisms may ultimately lead to improved understanding of genome-wide control mechanisms and the design of new pharmacological or genetic treatments for the benefit of patients.

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