Biological Cyprus

Trends and developments in biological research of the 21st century

Chief Editor
Andreas Ch. Hadjichambis

Editors
Demetrios Mappouras
Mikis Hadjineophytou
Panayiota Matsouka
Pavlos Neophytou
Maria A. Tsiarli
Soti Constantinou

Cyprus Biological Society

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2025

Cyprus Biological Society (CBS)

Chief Editor
Dr. Andreas Ch. Hadjichambis
Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus
Cyprus Centre for Environmental Research and Education, CYCERE Lemesos, Cyprus
Cyprus Biological Society, Thali 3, 2020 Nicosia, Cyprus

Editors
Dr. Demetrios Mappouras
Cyprus Biological Society, Thali 3, 2020 Nicosia, Cyprus

Mikis Hadjineophytou
Cyprus Biological Society, Thali 3, 2020 Nicosia, Cyprus

Panayiota Matsouka
Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus

Dr. Pavlos Neophytou
Cyprus Biological Society, Thali 3, 2020 Nicosia, Cyprus & Mendel Center for Biomedical Sciences, Kimonos 5, Egkomi, 2406 Nicosia, Cyprus

Dr. Maria Tsiarli
Molecular Biology, Cell Biology and Biochemistry, Brown University, USA

Soti Constantinou
Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus

ISBN: 978-9925-7814-3-0 (printed) ISBN 978-9925-7814-4-7 (electronic)

Cite this book:

Hadjichambis, A. Ch., Mappouras, D., Hadjineophytou, M., Matsouka, P., Neophytou, P., Tsiarli, M. A., Constantinou, S. (2025). *Biological Cyprus: Trends and developments in biological research of the 21st century* (p. 274). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

CYPRUS BIOLOGICAL SOCIETY
Georgiou Anastasiou 8, 3070, Lemesos, Cyprus
Tel.: 99 47 73 09, Fax: 25 33 56 82
E-mail: info@cbs.cy , Website: https://cbs.cy

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Preface

Cyprus is a country with highly qualified human resources, that ranks among the first countries in the European Union in holders of PhD and postgraduate qualifications. Cyprus is in the 5th place in terms of graduates of higher education. Biological research is part of the evolving Cypriot research and innovation ecosystem, an ecosystem that, while small, is constantly maturing and expanding, and is now ranked among Europe's Strong Innovators. Nowadays, biological research in Cyprus involves a competent critical mass of biological scientists, researchers, academics as well as universities, research institutes and also centers of excellence that allow it to look to the future with optimism. In addition, it covers a wide range of biological research in different fields, ranging from health to environmental and nature conservation.

This book is an anniversary edition in the context of the 45 years of the Cyprus Biological Society, which is one of the oldest scientific societies in the country. Many of the authors are Honorary Members of the Cyprus Biological Society and come from the academic and research field. The authors of the chapters of this book deal with a wide range of topics related to the biological research that is carried out today in Cyprus and abroad.

In the first chapter, the main university institutions, research organizations and research centres conducting biological research in Cyprus are presented. Furthermore, the strategic actions of the Cyprus Biological Society (CBS) for the promotion of biological research in Cyprus, are given, notably, by widening the participation base in CBS and by rewarding excellence in biology at all levels. Also, the chapter discusses the developments of contemporary biological research in Cyprus, which have been improved dramatically in recent decades, and also addresses its potential for further development in the future.

Chapter 2 details the evolution of sequencing technologies, revealing the mechanisms and limitations of the molecular sequencing techniques. It presents the fertile ground both in the field of clinical diagnosis and the evolving field of personalized medicine. The chapter also aims to reveal the implications of the application of sequencing methodologies both in biomedical research and in guidance at the clinical level. On the other hand, it discusses the undiscovered area relating to consumer-specified diagnostics that bears certain bioethical risks.

Chapter 3 is entitled “Fats that heal or Fats that kill”. It presents the effects of specific Omega-3 and Omega-6 polyunsaturated fatty acids on cardiovascular diseases. Supplementation with specific n-3 (omega-3) polyunsaturated fatty acids (PUFAs), primarily entailing eicosapentaenoic

acid (EPA) and docosahexaenoic acid (DHA), has been shown to reduce key risk factors of atherosclerosis, the main cause of cardiovascular diseases. The chapter summarizes the current clinical and molecular evidence on the effect of n-3 and n-6 PUFA supplementation in atherosclerosis and the role of the n-6/n-3 PUFA ratio in the optimum response to supplementation.

Chapter 4 focuses on untangling of the complexity of cancer by delineating the signaling mechanisms forging deadly alliances within the tumor microenvironment. In the chapter, the authors discuss the concept of the tumor microenvironment ecosystem with a particular focus on the contribution of the nervous system in brain and other solid cancers and the role of the Integrated Stress Response in promoting tumor cell adaptation to nutrient and hypoxia stress. The chapter outlines also some of the current and emerging concepts and future directions that shape novel approaches in the development of therapeutics for cancer.

Chapter 5 provides the insights of contemporary biological education in relation to sustainability and the socio-ecological issues which are fundamental components of biological literacy. The chapter also defines the Education for Environmental Citizenship and presents the pedagogical landscape, the characteristics, the notions and the competences of Education for Environmental Citizenship. In addition, it focuses at the details of the Education for Environmental Citizenship Pedagogical Approach for an innovative learning for achieving sustainability. Finally, the chapter describes a case study from Cyprus which facilitates important empirical data, using the ECQ, a duly validated questionnaire, already used in many international studies.

In Chapter 6, C. Kadis and his colleagues present the contribution of conservation biology research to the establishment and effective management of the Natura 2000 Network in Cyprus. This chapter presents the main outcomes of more than 20 conservation projects that have been carried out by the Department of Botany of the National and Kapodistrian University of Athens and the Nature Conservation Unit of Frederick University, in close collaboration with the competent authorities of Cyprus. The results of these projects have played a catalytic role in the delineation, operation, and effective management of the Natura 2000 network in Cyprus, while also contributing to raising public awareness about the role of the Natura 2000 Network and, more generally, about biodiversity conservation.

“Genetics of Inherited Endocrine Disorders in Cyprus: The present and the future”, is the focus of Chapter 7. All globally reported inherited diseases include a genetic connection with variable pathogenic status. This chapter provides information of current available data and future endeavors in Cyprus regarding inherited endocrine disorders. According to the chapter, currently

in Cyprus, several ongoing research programs look for the involvement of genomic, epigenetic and molecular factors, as well as the discovery of potential biomarkers for prognosis and diagnosis. The authors, pointed out that it is certain that innovative approaches will be applied in the clinical practice in Cyprus health care system with certain benefits for patients with rare endocrine disorders.

C. Deltas and his colleagues, in chapter 8, present the Biobank of Cyprus as a new prospect for next generation biomedical research in Cyprus. Biobanks are organized collections of medical records and biological material, including DNA, aimed at enabling precision medicine. Serving as research infra-structures, Biobanks provide high-quality data and materials for clinical trials, epidemiological studies, and genetic research. They facilitate the discovery of new biomarkers to achieve accurate diagnosis, prognosis, and disease prevention, as well as the discovery of innovative disease-specific therapeutics. As a key pillar of the Centre of Excellence for Biobanking and Biomedical Research, the Cyprus Biobank fosters collaborations amongst the scientific community and volunteers (patients and healthy individuals), contributing to an all-round improvement of life quality for everyone.

Lastly, Chapter 9, presents the significant amount of research data concerning the conservation biology of the endemic, rare, and threatened plants of Cyprus that have been accumulated during the past three decades. Most of these data were acquired through scientific work carried out by the Department of Botany of the National and Kapodistrian University of Athens (Greece) and the Nature Conservation Unit of Frederick University (Cyprus), in cooperation with the Department of Environment, the Department of Forests and the Agricultural Research Institute of the Ministry of Agriculture, Rural Development and Environment of Cyprus. These scientific projects focused mainly on seed biology and reproductive effort of the targeted plants and the acquired knowledge was utilized for *in situ* and *ex situ* conservation actions.

Obviously, we do not think that this scientific book can present the multifaceted biological research that is currently being carried out in Cyprus, in its entirety. However, it can be a useful tool for the contemporary scholar of biological research in Cyprus. We hope that this book will be a springboard for a more focused, more substantial and more generous promotion of biological research in Cyprus, which will give Cyprus a competitive advantage and improve the quality of life, health and environment of our country.

Dr. **Andreas Ch. Hadjichambis**
President of the Cyprus Biological Society

Chapter 1
Introduction to contemporary biological research in Cyprus

Andreas Ch. Hadjichambis

Cite this chapter:

Hadjichambis, A. Ch. (2025). Introduction to contemporary biological research in Cyprus. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 17-24). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 1
Introduction to contemporary biological research in Cyprus

Hadjichambis Ch. Andreas

Cyprus Biological Society (CBS) & Cyprus Centre for Environmental Research and Education (CYCERE), Lemesos, Cyprus & Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus

Keywords: Biological research, Cyprus, scientific breakthroughs, biological breakthroughs.

Chapter Abstract This chapter briefly reviews the development and achievements of biological research and presents the main scientific breakthroughs related to biology in the last two years. As it can be seen, every year, many of the scientific discoveries are in biological research or related to the research field of biology. The main university institutions, research institutes and research centres conducting biological research in Cyprus are also presented. Furthermore, the strategic actions of the Cyprus Biological Society (CBS) for the promotion of biological research in Cyprus, are given, notably, by widening the participation base in CBS and by rewarding excellence in biology at all levels. Also, by promoting biological research in various ways such as organizing and supporting scientific conferences, workshops, seminars and events related to biological research. The Special Scientific Committees created by the Cyprus Biological Society (CBS) also contribute to this. Contemporary biological research in Cyprus has improved dramatically in recent decades and has the potential for further development in the future. All branches of biological research from health to ecological and environmental fields can offer work positions and opportunities for a successful career in biological research.

1.1 Introduction

Some 370 years after the discovery of the microscope by Antonie van Leeuwenhoek, biology has become a modern and rapidly evolving science, a science of the 21st century. Today, the rapid development of biological research presents applications in health, the environment, the economy and society at large. Biological research is of vital importance for a contemporary society, since innovations emerging from this research can contribute to economic development in the branch of bioeconomy and can contribute also to improve the health and quality of life of citizens. Europe has already celebrated 10 years of the bioeconomy revolution based on Organic Europe. Nowadays, the bioeconomy sector in Europe represents over 5.7 billion euros of public and private investment. The revolution of biology, therefore, is here both in Europe and internationally.

Biology is the science of our century and the main protagonist of the 4th industrial revolution whose threshold we have already crossed. Interconnectivity, intelligent automation, the internet of things, the internet of systems cannot be realized without the essential contribution of biology. We are already talking about transhumanism, where the improvement of cognitive and other human skills and abilities, are attempted as well as genetic engineering and the in vivo clinical application of the CRISPR molecular scissors for the correction of gene errors. New generation "intelligent" innovative drugs, as a result of biological research, are already circulating worldwide for the gene therapy of diseases. Neurological repair and genetic engineering are booming. We live in the era of biology, and personalized precision medicine, which is the future of medicine, cannot be achieved, naturally, without the essential contribution of our science, biology.

1.2 The recent developments of the science of biology

This rapid development of the science of biology can be seen, at an international level, through the most important scientific discoveries of 2023, according to "Science" magazine Cantwell (2024). Five (5) of the top 10 scientific breakthroughs of 2023 are related to biology. These are: (1) GLP-1 therapies, where clinical trials have shown that these therapies, which were known to treat diabetes, offer additional benefits in combating obesity and other health benefits. (2) The discovery of human footprints, through innovative sperm chronology, in White Sands National Park was another major discovery that pushed back the arrival of humans in the Americas by 21,000 to 23,000 thousand years. (3) Malaria vaccines are ready for widespread use and will help reduce child deaths and serious health problems. (4) In the field of environmental biology, the great slowdown of the Earth's carbon pump was discovered. Antarctica's ultra-salty waters are absorbing heat, oxygen and carbon dioxide at slower rates than before. (5) Finally, small, but significant progress, has been made against Alzheimer's disease since new drugs, such as Lacenemab, were approved that are believed to slow the progression of the disease.

Year 2022 was an equally exciting year for biological research. Based on the "Science" scientific journal Cantwell, (2023), seven (7) of the most important scientific breakthroughs are related to biology and this makes us proud as biologists. (1) The perennial rice that was created and that promises easier cultivation is a fact. Researchers in China have shown that perennial rice can be a benchmark and save farmers from many weeks of difficult and laborious work. It's called Perennial Rice 23 (PR23), and it took more than 2 decades of biological research to improve its yield and quality. (2) The scientific breakthrough of the year for 2021 was Artificial Intelligence tools that predict the three-dimensional structure of proteins from the sequence of their building blocks, their amino acids. Extending this work, researchers have now used artificial intelligence to design entirely new proteins that could be used in vaccines, building materials or nanomachines. One technique, called "hallucination," starts with random sequences and mutates them into sequences that are sure to fold into stable proteins. (3) A third discovery, the discovery of a giant bacterium of enormous size, stirred biology this year, challenging traditional definitions of bacteria. Germs are supposed to be microscopic, but this bacterium, named *Thiomargarita magnifica*, can be 5,000 times larger than many bacterial cells and is about the size of a thumbtack. (4) The fourth major biological breakthrough of 2022 is the large-scale clinical trials of two new Respiratory Syncytial Virus (RSV) vaccines that have finally proven they can safely protect the two groups most affected by this common infectious disease: infants and the elderly. (5) Epstein-Barr virus, the herpes virus, appears to be associated with Multiple Sclerosis (MS) which afflicts 2.8 million sufferers worldwide. The findings may lead to new ways of treating or preventing this muscular disease. (6) A protective ERAP2 gene variant was identified in Europe during the Black Death pandemic and is thought to have protected people carrying this variant in their genome. It could be the strongest example of natural selection in the human genome. However, this protection seems to come at a price: The same variant carries a higher risk of developing autoimmune diseases such as Crohn's disease and rheumatoid arthritis. (7) Finally, the reconstruction of an ancient ecosystem from DNA of 2 million years was also important in 2022. Biologists have managed to extract microscopic fragments of DNA at least 2 million years old from frozen ground in the Arctic.

1.3 Biological research in Cyprus

In Cyprus today we have a number of universities, public and private, that provide branches of life sciences and biological research. Among them are the University of Cyprus, Cyprus University of Technology, European University Cyprus, University of Nicosia, Frederick University. ~~and others.~~ Also particularly encouraging is the presence and activity of the Department of Fisheries and Marine Research, the Cyprus Agricultural Research Institute. In addition, other independent research institutes related to biology exist in Cyprus, such as the Cyprus Institute, the newly established Cancer Institute, the Center of Excellence for Biobank and Biomedical Research, the Cyprus Institute of Neurology and Genetics which has already

completed 34 years of life. Also, other research institutes carrying out research related to biology are the Cyprus Marine and Maritime Institute, the Cyprus International Institute for Environmental and Public Health at the Cyprus University of Technology, and the Cyprus Center for Environmental Research and Education – CYCERE. These universities and research institutes are participating with their research partners in the forefront of the most innovative and competitive research projects in Europe and worldwide.

As the Cyprus Biological Society, we have set as our priority the promotion of biological research in Cyprus as well as the promotion of Biology in general in the Cypriot community and beyond. For this purpose, we have adopted a series of policies and actions aimed in this direction:

1. Broadening the participation base in Cyprus Biological Society of (CBS). CBS significantly expanded its participation base by increasing the number of its members by more than 25%. Of these members: (a.) 1/3 comes from the academic sector, university professors and researchers in universities and research centers. (b.) over half come from secondary education. (c.) 1 in 6 are biologists from the private sector. (d.) others active in ministries and the wider public sector.

2. Awarding "Excellence in Biology" at all levels. The Cyprus Biological Society (CBS) encourages and rewards excellence in biology at all levels. From the renowned scientists - researchers and academics, to biology students, to students who distinguished themselves in the biology Olympiads and to our educational partners who are members of the Coordination/Advisory committees of the six (6) Biology Olympiads we organize. In this way we look forward to increasing biology excellence and spreading it to more and more citizens and especially to our young people who are the future of this country. At the level of students, CBS has proceeded to establish, announce and award the CBS Awards for Undergraduate Biology Students "Christos Georgiadis", for Postgraduate Biology Students "Ouranios Ioannidis" and the CBS Award for Doctoral Biology Students "Kyriakos Georgiou". At student level, six (6) Pan-Cypriot biology Olympiads have been established and are organised every year in all middle school and high schools in Cyprus. Also, CBS selects and sends entries with the Cypriot delegations to the International Olympiads such as the International Biology Olympiad (IBO), European Olympiad for Experimental Sciences (EOES) and International Jounior Science Olympiad (IJSO).

3. Promotion of the biological research through several events. CBS organises and supports scientific conferences, workshops, seminars and workshops related to biological research. In addition, it has established an "Excellence in Biology" lecture series, where it hosts scientists and academics of international repute and esteem from all over the world. Furthermore, it publishes several scientific policy positions for biological issues important for the enhancement of biology.

4. Specific scientific committees. CBS has created Special Committees for thematic areas where it promotes various actions. Specifically, it has created the Committees:

- The Health Committee of the Biological Society of Cyprus (CBS) aims to strengthen the contribution of Biology and Biologists in the field of Health.

- The Committee of the Environment of the Cyprus Biological Society (CBS) aims to strengthen the contribution of Biology and Biologists in the field of Environment and Nature Protection.
- The Committee of Secondary Education and Olympiads of Biological Society of Cyprus (CBS) aims to strengthen the contribution of Biology and Biologists to Secondary Education and the International and National Olympiads.
- The Higher Education and Research Committee of the Biological Society of Cyprus (CBS) aims to enhance the contribution of Biology and Biologists in the field of Higher Education and Research.

1.4 Conclusions

In conclusion, contemporary biological research in Cyprus has been enormously enhanced during the last decades and it has the potential for further developments in the future. All branches of biological research from health, up to ecological and environmental fields, can offer employment positions and opportunities for successful carriers in biological research.

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Chapter 2

Nucleic Acid Sequencing: From the Sanger Method to Massively Parallel Sequencing and Modern Applications of Molecular Diagnostics

Marios A. Diamantopoulos, Panagiotis Adamopoulos, Emmanouil G. Fragoulis and Andreas Scorilas

Cite this chapter:

Diamantopoulos, M. A., Adamopoulos, P., Fragoulis, E. G. and Scorilas. A. (2025). Nucleic Acid Sequencing: From the Sanger Method to Massively Parallel Sequencing and Modern Applications of Molecular Diagnostics. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 25-63). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

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Chapter 2
Nucleic Acid Sequencing: From the Sanger Method to Massively Parallel Sequencing and Modern Applications of Molecular Diagnostics

Marios A. Diamantopoulos, Panagiotis Adamopoulos, Emmanouil G. Fragoulis and Andreas Scorilas

Department of Biochemistry and Molecular Biology, Faculty of Biology, National and Kapodistrian University of Athens, Panepistimiopolis, 15701 Athens, Greece.

Keywords: Nucleic Acid Sequencing, Sanger Method, Massively Parallel Sequencing, NGS, Molecular Diagnostics, Molecular Biomarkers, Precision Medicine.

Chapter Abstract The complete sequencing of the human genome has been a turning point in biology and has provided enormous insight into genetic information. The progress from the early days of single sequencing according to the Sanger method to the beginning of the era of molecular techniques of massively parallel sequencing was a major "revolution" that radically changed the course of the biomedical sciences. This chapter details the evolution of sequencing technologies, revealing the mechanisms and limitations of these molecular sequencing techniques. The latter find fertile ground both in the field of clinical diagnosis and in the evolving field of personalized medicine. The chapter aims to reveal the implications of the application of sequencing methodologies both in biomedical research and in guidance at the clinical level. On the other hand, the undiscovered area relating to consumer-specified diagnostics also bears certain bioethical risks. This issue needs constant dialogue, their critics, and holding the same ethical standards across all genetic research and care. The curriculum of the nucleic acid sequencing technologies course, which focuses on the personalized medicine concept, is essential for the reason that it projects the testing instruments as diagnostic tools that assist in the design and implementation of prevention strategies intended for the early treatment of various diseases and lastly give in-depth information on the features of genetic mutations.

2.1 Introduction

The human genome sequence, that emerged at the start of the twenty-first (21st) century, marked a profound confluence in the history of the biological sciences, sparking a revolution underpinned by extraordinary progress in nucleic acid sequencing technologies (Hood & Rowen, 2013). Over the past few years, high-throughput sequencing methods have advanced quickly, moving from the Sanger method to third generation sequencing. New features distinguish each of the next generation sequencing methods. This fact opened up an era in which the staggeringly complex code of human genetics was unveiled, revealing many biological insights and creating a new scientific frontier (Heather & Chain, 2016).

The Sanger method – the bedrock of DNA sequencing since the earliest days – has given way to a brave new world that has been dominated by massively parallel sequencing (Bruijns, Tiggelaar, & Gardeniers, 2018) as these technologies have enabled, and been driven by an extraordinary wave of groundbreaking genetic discoveries. The method displayed the complicated symphony of genetic data in greater detail and accuracy, ranking among the most outstanding cornerstones in the hall of fame of molecular biology (Eren, Taktakoglu, & Pirim, 2022). This venerated technique saved for its structured, sequential data gaining has been heralded as one pivotal epic in scientific history—a fundamental reconceptualization of how the intricate genome is instituted. In its painstaking but exacting methodology, like an artisan blueprinting genetic data, the Sanger Method would provide the first scaffolding to unravel this mysterious language that DNA encodes (Crossley et al., 2020; Shendure & Ji, 2008).

Next and Third Generation Sequencing (NGS/TGS), a common term that refers to the application of massively parallel sequencing, sounded one of the most revolutionary points in the history of genomic analysis (Koboldt, Steinberg, Larson, Wilson, & Mardis, 2013). This revolutionary breakthrough has taken the entire field to new unimaginable heights marked with an impossibility of parallelism not only in characterization but also in range, thus underlining a new era characterized by a swift life span; however, it is high throughput and unimaginably cost-effective nature (Tucker, Marra, & Friedman, 2009). Both NGS and TGS methodologies are distinguished from Sanger sequencing by their ability to implement massive parallel sequencing, up to hundreds of million DNA fragments. These features have not only dramatically boosted the throughput of the sequencing data but also have notably decreased the sequencing cost. These developments have made large-scale omics investigations, including the sequencing of the complete human diploid genome, both technically and financially feasible. The classification of high-throughput sequencing technologies is still up for debate. However, it can be broadly classified into two groups: next-generation sequencing (NGS), also known as second-generation sequencing and third-generation sequencing (TGS) technologies. Next-generation DNA sequencing technologies have taken genetic analysis far away from the

conventional workflows, allowing the comprehensive and rapid exploration of the vast expanse of the genetic code (Satam et al., 2023). This groundbreaking advance in technology has revolutionized the throughput of gene analysis (Day-Williams & Zeggini, 2011). Currently, the various sequencing generations need a consistent definition. Nonetheless, in this chapter TGS is differentiated from NGS due to the characteristic of single DNA molecule sequencing (Thompson & Milos, 2011).

This chapter sets out an ambitious and expansive odyssey that could effectively unravel the rich tapestry of the evolutionary trajectory that has carved out the landscape of nucleic acid sequencing methodologies. It carefully shepherds the reader through the contorted pathways that thread through these approaches' historical evolution, seminal turning points, and modern day relevance (Akintunde, Tucker, & Carabetta, 2023). Through examination of this journey through time, the reader will gain insight into the profound impact and pervasive significance that nucleic acid sequencing has carved out as it has wended its way from a scientific quest to a preeminent methodology for interrogating biological systems and to a compass that now steers both biomedical research and clinical applications. The following material assumes the somewhat ambitious task of retracing the evolutionary trajectory of methods for nucleic acid sequencing back through time. However, now it is with a special mission to bring out the exact mechanisms, subtle powers, and associated limitations intrinsic to every sequencing paradigm. In so doing, it seeks to delineate the historical curve wherein lies the evolution of these methodologies, unfolding for readers the transformative milestones that have sculpted this landscape of genetic analysis. Without an outline of the vast field of molecular diagnosis, this chapter highlights that these sequencing methodologies are indispensable tools driving the makeover of the landscape of healthcare services delivery (Sanger, Nicklen, & Coulson, 1977).

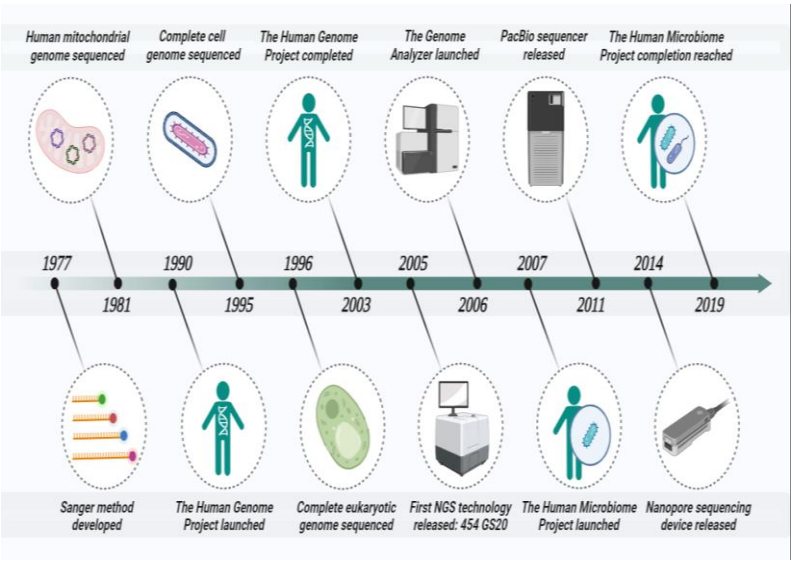

Fig. 2.1 The history of DNA Sequencing.

From their pivotal role in diagnosing illness, prognosis, and therapeutic stratification to implications in personal medicine with genomics burgeoning incorporation, these technologies resonate profoundly in multiple facets across health (Weber, 2015). Drawing on much more than mere historical annotation, this intensive study exposes the substantive implications and enormous consequences that methodologies of sequence determination continue to have in shaping the flow of biomedical research and directing clinical practice (Fig. 2.1).

2.2 Evolution of Sequencing Techniques

2.2.1 The Sanger Method: Foundation of Sequencing

The development of sequencing has provided a pioneering base in an attempt to elucidate details pertinent to the human genome's genetic constitution. It followed contributions made by Frederick Sanger in 1975 and took on commercial form in 1977 (Zimmermann, Voss, Schwager, Stegemann, & Ansorge, 1988). Sanger established the principle and further developed the original technique, taking in consideration chain terminating dideoxynucleotides to visualize the behavior of the complex human genome (Sanger et al., 1977). his invention was developed and discovered at the end of the 1970s, being one of the revolutionary steps in genomics—one more technically advanced invention in the sequence of DNA (Giani, Gallo, Gianfranceschi, & Formenti, 2020).

Although aiming at perfection by formulating a new procedure, the Sanger method itself also inherited certain innate limitations that hindered in-depth utilization. The method was labour intensive and needed gel electrophoresis and radioactive labelling. What bothers most is the high cost per base pair sequenced and a lengthy procedure that can be applied to genomic scale projects and routine sequencing programs (Akintunde et al., 2023), though these limitations can be overcome in the future.. The output from capillary electrophoresis has been proven to be highly efficient in generating automated DNA sequence data and has become the standard on which genomics is being made today. Limited by the resolution of experimental procedures, this technique provided a basis that has sparked further innovations and the relentless development of new ideas. Finally, this scientific domain had been far beyond what people previously considered in genomic explorations (Maxam & Gilbert, 1977).

The basis of the method is underlined by two fundamental processes in enzymatic DNA replication, from the idea of chain termination that controls the art of complicated sequencing. It involves an interruption strategy at a particular site of the process of synthesis of DNA by including in the replication process dideoxynucleotides (ddNTPs) (Fig. 2.2). These nucleotides have been modified at their 3'-OH group so that there is no longer a position along the DNA strand for the next incoming nucleotide to bind and create the phosphodiester bond. Thus, in case a ddNTP is used to

synthesize the DNA, this bonding will occur, but any further progression of the DNA synthesis will be terminated at this specific site (Maxam & Gilbert, 1977). The strategic use of ddNTPs in the Sanger Method assures that replication of DNA is bound to stop at a particular base. As a result, a collection of DNA fragments is generated and shortened at random nucleotide sites, generating a mix of strands with different lengths (Sanger & Coulson, 1975).

An essential part of the process is that it incorporates gel electrophoresis in determining the newly assembled proteins. Thus, depending on different lengths, the selective technique separates those shortened DNA fragments terminated by ddNTP incorporation. It relates to a process in which DNA fragments move through a polyacrylamide gel under an electric field, with shorter molecules moving with incredible speed and displacement from long ones. Such separate DNA fragments by size allow individual parts to be recognized. Following exposure to electrophoresis on a polyacrylamide gel, the fragments from each sequencing reaction usually separate into discrete bands within four parallel lanes. Based on the fragments' migration in the gel, base fragments can then be determined, thus allowing for a very detailed base pair analysis. The lengths of all the DNA fragments added up, thereby giving an exact determination of the DNA sequence and thus enabling researchers to translate the genetic information in the DNA template (Hultman, Stahl, Hornes, & Uhlen, 1989; Ueberfeld, El-Difrawy, Ramdhanie, & Ehrlich, 2006). This protocol was further modified to automate the sequencing process, as described by Rosenthal and Charnock-Jones (Rosenthal & Charnock-Jones, 1992). One such modification during the years was the development of dye-terminator sequencing. The most important advantage of the method is that it is highly accurate and fast.

One such modification during the years was the development of dye-terminator sequencing. The most important advantage of the method is that it is highly accurate and fast. Applied Biosystems, Inc. (ABI) introduced the first automated platform for DNA sequencing in 1987—the ABI model 370, developed by Leroy Hood and Mike Hunkapiller. This machine could produce base pairs (bp) read lengths up to 350 per lane. Then, in 1995, ABI developed a new model that proved part of the solution: the ABI PRISM 310 Genetic Analyzer. In a sequencer called the capillary sequencer of Swerdlow and Gesteland, they put slab gels to the polyacrylamide gel-filled capillaries. Now sequencers with 4, 16, 48, 96, or even 384 capillaries are on sale. Increased throughput sequencing with longer read lengths increases the expansion of capillaries (Franca, Carrilho, & Kist, 2002). The Sanger method was revolutionary for its time but limited to a sequential design, and therefore, the scale-up issues had to be addressed.

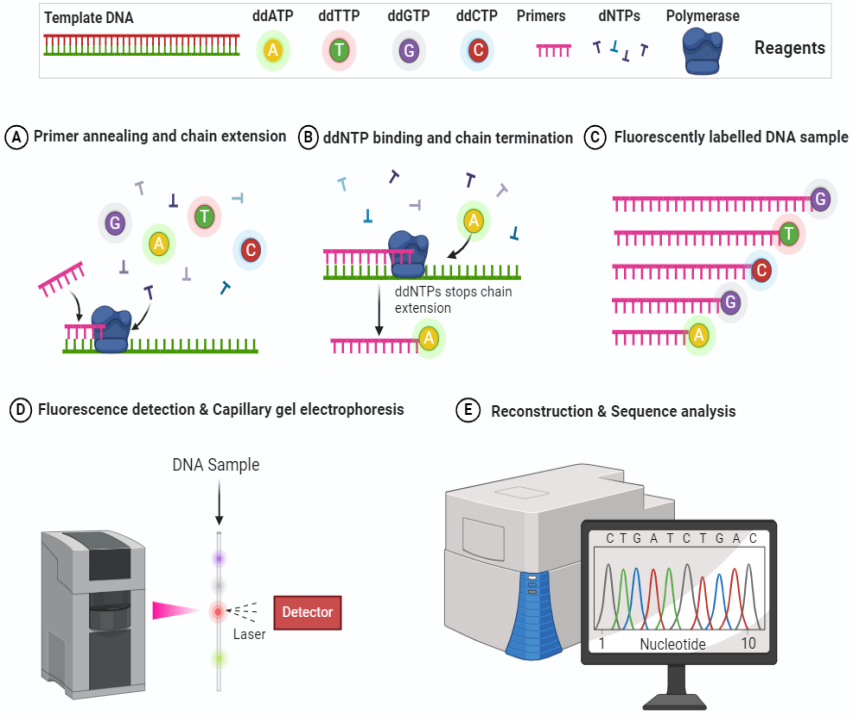

Fig. 2.2 Process of Sanger Sequencing

2.2.2 The Move toward Next-Generation Sequencing (NGS)

The sequencing methodology was revolutionized due to next-generation sequencing (NGS) approaches that allowed the genetic material sequence determination to be more feasible (von Bubnoff, 2008). The passing of the time hitherto broadly speaks of the commonly used rapid and low-cost technologies to put an end to their slow companions. They have improved on the bottleneck that requires first-generation methods to prepare individual DNA templates. In addition, when armed with sophisticated software and toolsets from such other disciplines as bioinformatics and computational biology, these technologies make a great swell in data generation (Metzker, 2005). The main drive for such a revolutionary phase was the advent of cheap and fast sequencing technologies, which paved the way into the current era of NGS technologies (Metzker, 2010).

A radical leap was mandating one to break from traditional methodologies for sequencing into a domain characterized by speed and affordability in the analytics of genomics. NGS technologies have played the leading role since there was a profound change and have made the milestone in the genetic exploration through revolution it was had. Among the ancient ways that can be covered by NGS are novel and trails making techniques of pyrosequencing, solid sequencing, and illumina

sequencing, having a large part of the process falling on the user. This led to a staggering degree of parallelization, its magnitude so big that it marked the onset of a completely different era for high-throughput sequencing, one that would eventually overcome the earlier constraints of such sequential methodologies (Goodwin, McPherson, & McCombie, 2016). A large number of projects that covered millions of bases in the genome were launched because of this spurred-on throughput, which massively reduced the cost per sequencing sample. NGS methods are pushing heavily against the traditional and narrow boundaries of sequencing occupying the not only a significant but also a breakthrough place in next gen technologies due to the promptness and extensiveness of results produced by the opened space in the near future (Shendure & Ji, 2008). MPS technologies have caused a new revolution in the modern landscape. Like all the other genomic tools, the current sequencing technologies have seen a paradigm shift; NGS technologies have emerged as the most daring strategies for change to completely remake the look of a parallelized fragmentary landscape. Such advances that represent methods such as those represented by Illumina sequencing forecast progressive methodologies, with the capability of structurally transforming the face of the sequencing landscape through massively parallel processing (Mardis, 2008b).

Illumina sequencing technologies mostly need the procedure in which DNA has to be shattered into easy-to-handle tiny pieces to be employed. These broken DNA fragments become captured in a filter by a process called clonal amplification. During this process, each fragment is copied countless times on a solid flat surface, resulting in millions of clusters. Where Illumina sequencing (Fig. 2.3) shines is how these few millions of clusters are treated independently in every single run, with each cluster going through a sequencing process that is sequential in nature. This results in a significant increase in the rate of sequencing which is a problem faced by conventional ways of detection and segregation. Conversely, this technology revolutionizes genomics to an instant and exceedingly profound stage that had not been envisioned prior (Bentley et al., 2008).

In terms of second generation platforms like Illumina sequencing, they work with reliability owing to the fact that they utilize the partially dissociable terminator and fluorescent label technology. These technologies pause at a certain gap in the DNA strands, carefully replicating and adding new nucleotides while keeping track of every step along the way. Reversible terminators give researchers the ability of nucleotide addition sets and the signals that show which nucleotides are inserted. This approach guarantees the signals are captured and saved accurately for the processes of nucleotide incorporation's each step, which helps obtaining the DNA sequences. Through the combining of the signals from each newly added nucleotide, the annotation of the template molecule are obtained, giving the ability to recreate accurately the genetic code (Adey et al., 2013). The two things that distinguish NGS platforms from the preceding generation of sequencing technologies is the improvement in performance and the ability to do it more affordably. They continue to make advancements with the application of precision sequencing and sequencing in parallel

that drive the new era. Such approaches help in discovering novel aspects of genome complexity which can then allow for its deciphering at high speed, precision, and depth level, drastically changing the previously existing methodologies of genomics and biomedical research. These forms of DNA sequencing were not perfect, but they provided a platform for these breakthrough developments, with Ion Torrent as an example seeking to surpass others by means of its high throughput lifestyle.

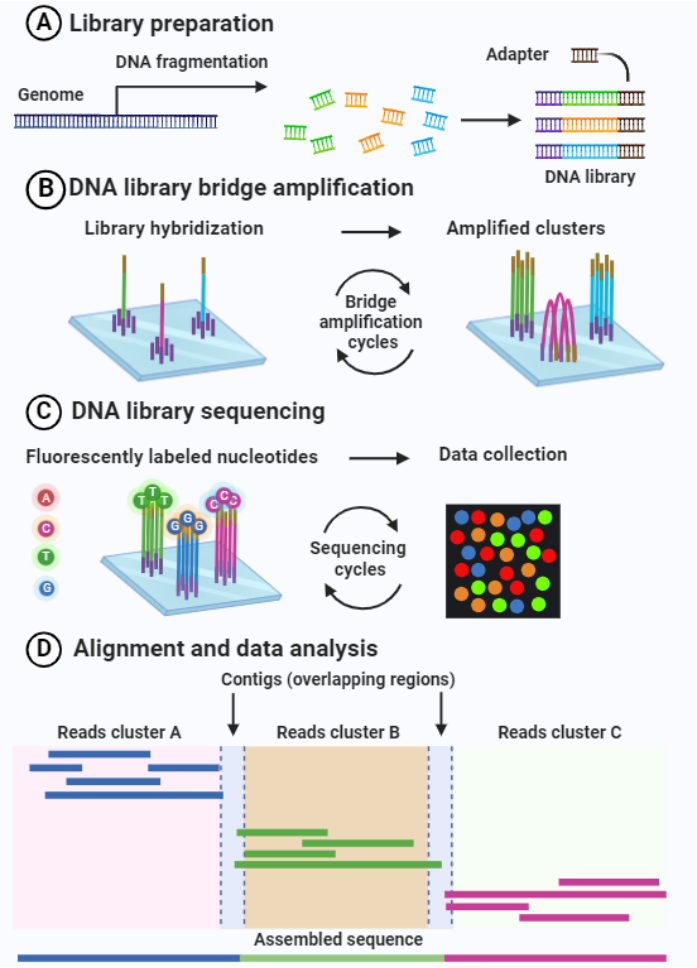

Fig. 2.3 Next Generation Sequencing (NGS) workflow (Illumina sequencing)

The Ion Torrent sequencing technology is characterized by a unique chemistry. During the reaction, the DNA polymerase enzyme assists in building a DNA strand by forming covalent bonds. This process releases pyrophosphate and a proton, leading to a decrease in the surrounding pH, which serves as a signal for detecting the developing DNA sequence (Hui, 2014; Rothberg et al., 2011). In this sequencing apparatus, each tiny well on a semiconductor chip holds a single-stranded template

DNA molecule that has been amplified and is ready for sequencing. These wells are filled with DNA polymerase and unmodified deoxynucleoside triphosphates (dNTPs) in a specific order (Eid et al., 2009). When the proper species of the dNTP is used, a biochemical reaction commences only when the nucleotide base matches the one that will be installed on the growing DNA strand. This reaction leads eventually to the production of hydrogen ions. These ions are then captured by the ISFETs. When pH becomes altered, the change in the potential of electrical movement may be recognized to determine the amount of hydrogen ions produced during every particular stage. Upon every stage, the presence of a dNTP is removed, and a longer chain of a primer is made by using the rest of bases, which are dNTPs. And thus the next type of dNTP for the incorporation continues the process. This approach is popularly used in NGS, which employs it to its great flexibility positioned by an algorithmic unfolding and application-specific uniqueness providing certain benefits (Fig. 2.4).

Many technologies (e.g. Illumina, Ion torrent) are involved in NGS (Harrington, Lin, Olson, & Eshleman, 2013; Meyer & Kircher, 2010, Hu, Chitnis, Monos, & Dinh, 2021). They are considered the most suitable choice for whole-genome, exome, and RNA sequencing due to its high accuracy and rapid data throughput (Datta, Datta, Kim, Chakraborty, & Gill, 2010). NGS methodologies have democratised the genomic research field for anybody globally (Goodwin et al., 2016). The current transformation experienced is one with more magnitude and variety in different processes that are gene-based and include large projects that are scaled in terms of population genetics, studies on cancer genomics, and metagenomics, among others. In fact, besides research domains alone, it has more recently infiltrated the clinical context regarding diagnostics, personalized medicine initiatives, and decoding genetic susceptibilities to diseases (Satam et al., 2023).

The advancement of Next-Generation Sequencing (NGS) extends beyond hardware improvements, relying heavily on bioinformatics tools and computational analyses, which are fundamentally important. These tools are utilized for data management and the analysis of large volumes of data obtained from sequencing. They play crucial roles in tasks such as sequence alignment, identifying genetic variants, detecting structural variations, and interpreting complex genetic information (Pereira, Oliveira, & Sousa, 2020). Despite significant advances, the NGS technique still faces several ongoing challenges. These include areas such as data management and analysis, standardizing procedures, implementing quality control measures, and addressing ethical considerations regarding data privacy and consent (Martinez-Martin & Magnus, 2019). NGS represents a technological breakthrough with the potential to revolutionize genomics, impacting everything from basic scientific research to medical applications. Its ongoing evolution holds promise for further advancements, accelerating discovery and progress in genomics and biomedical science (Goodwin et al., 2016).

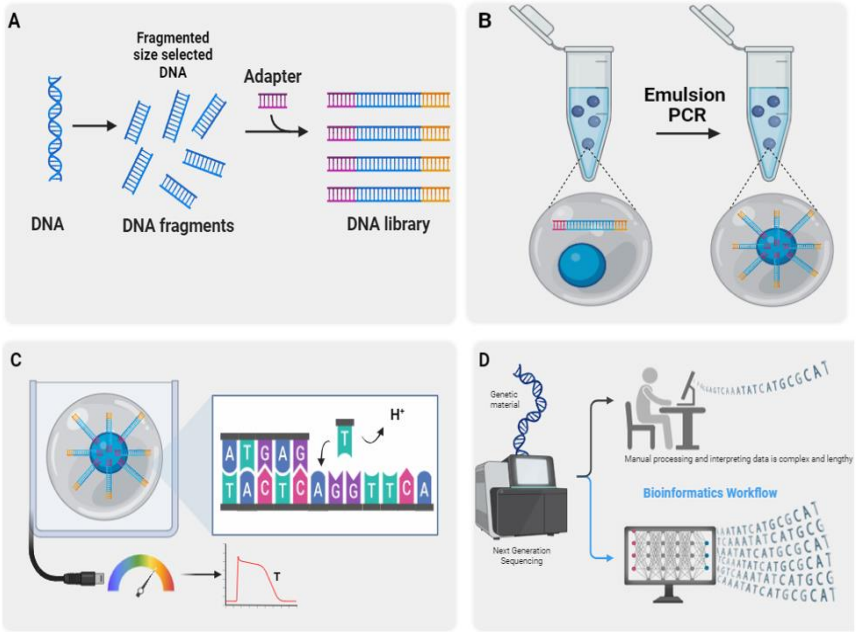

Fig. 2.4 Ion sequencing work flow. The overall workflow is shown in (a). A genome library is prepared by fragmenting and size-selecting DNA, followed by the ligation of forward and reverse adapters (b). Each adapter-ligated template is clonally amplified onto a bead, so that each bead contains many copies of the same DNA template (c). Sequence on the chip, sequencing primers and DNA polymerase are then bound to the beads, which are pipetted into wells on the chip (d). The chip is then repeatedly flooded by nucleotides, which, when binding to the complementary nucleotide on a template, release an ion. At each flow, the electrical signal at each well is measured, indicating the number of incorporations.

Recently, another NGS technology from MGI Tech has been introduced to the scientific community. This methodology involves template preparation using nanoballs, which are densely packed DNA clusters attached to spherical beads. The process begins with DNA fragmentation, where the DNA sample is broken into small fragments. These fragments are then ligated with adapters, which contain sequences necessary for the DNA circularization (nanoball) as well as for the subsequent steps of sequencing. Following adapter ligation, the DNA fragments are amplified through the emulsion PCR to generate clusters of DNA fragments on the surface of the nanoballs. The nanoballs serve as solid supports for the DNA clusters during sequencing. This template preparation method with nanoballs ensures efficient and high-density sequencing, enabling accurate determination of the DNA sequence. MGI Tech offers several sequencing platforms that support various research needs and throughput requirements. Up until today, the prominent MGI sequencing platforms include DNBSEQ-T7, DNBSEQ-G400 and DNBSEQ-G50, etc. These platforms leverage innovative sequencing technologies developed by MGI Tech, empowering researchers with reliable and efficient tools for exploring the intricacies of genomics and beyond (Fig. 2.5).

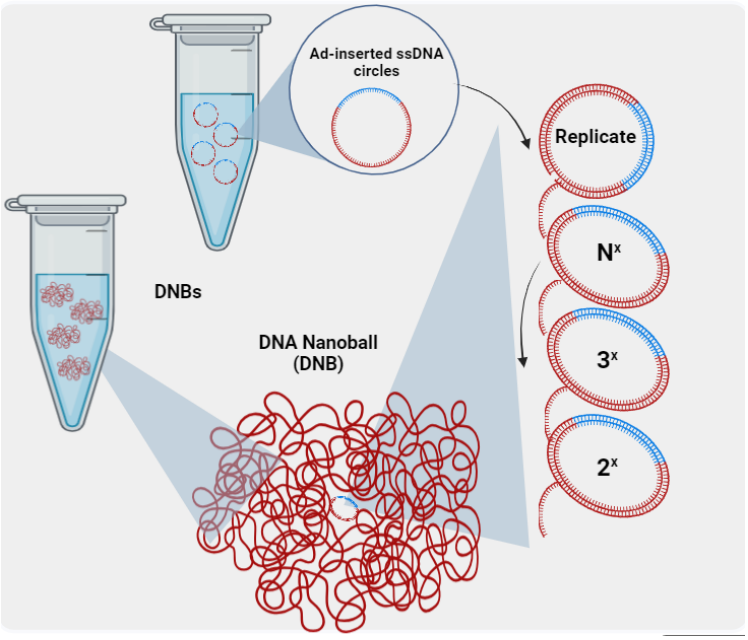

Fig. 2.5 Nanoballs from MGI Tech workflow

2.2.3 Third Generation Sequencing (TGS): Shaping the Contemporary Era

These technologies (aforementioned) in combination (X.2.2) revolutionalized genomics and now allow large-scale parallel generation of biomedical data. A next-generation technology is coming into existence every day due to the crisis of the never-ending quest for effectiveness. TGS exploit single-molecule sequencing (SMS) methods consequently getting rid of the biases and drawbacks of conventional polymerase chain reaction (PCR) (Rhoads & Au, 2015). Some lines of demarcation cannot always be drawn very quickly between second- and third-generation approaches, for approaches described in this chapter have brought along improvements for throughput and read lengths, eliminating the need for clonal amplifications for better efficiency of the sample preparations, hence saving time and costs of sequence analysis (Pareek, Smoczynski, & Tretyn, 2011). Massive Parallel Sequencing (MPS), often called high-throughput sequencing, represents the peak of evolutionary progress in sequencing techniques (Goodwin et al., 2016). This advancement heralds a sequencing revolution unlike anything seen before, unlocking unprecedented sequencing possibilities. Its innovative design enables the sequencing of billions of DNA fragments simultaneously, setting new standards for others to aspire to.

However, TGS technologies, by many orders of magnitude, have transcended the point to which conventional methodologies previously tethered mainstream genomic

analysis and have taken a seismic leap, surmounting limitations and charting new frontiers. Devices from Helicos BioSciences (Milos, 2008), Pacific Biosciences (White & Hesselberth, 2022) and Oxford nanopore (Lu, Giordano, & Ning, 2016) are at the forefront of this evolutionary leap, extending the sequencing limits. The availability of such state-of-the-art technologies has added to the armoury of sequencing tools, which were presumed nearly impossible to exist earlier. It has become possible to sequence single DNA molecules in real-time. The incredible advancements in accuracy and the ability to read longer sequences have greatly enhanced our understanding of the intricate details of the genome. These breakthroughs have shed light on aspects of genomic analysis that were previously inaccessible, providing deeper insights into the complexities of genetic information. The unprecedented development in TGS technologies has restructured the peripheries of genomic research and opened the door for researchers to explore previously unknown or unknown frontiers in several scientific sectors. These advances make it possible to effectively study in-depth genetic environments and open an era for studying science anew (Loman & Quinlan, 2014).

New high-throughput sequencing techniques are personified by groundbreaking technologies such as tSMS (Milos, 2008), PacBio (Xie et al., 2020), and Oxford nanopore (Harris et al., 2008),. These technologies are free of limitations that have defined conventional methods for years. They use unique mechanisms that fundamentally change the character of all the sequencing proceedings, leading to unexplored potential in genetic analysis and exploration (Jain, Olsen, Paten, & Akeson, 2016a). For example, the emerging technologies like the Oxford Nanopore and the Pacific Bio adopt the approach of fresh thinking for the betterment of the conventional sequencing by employing the innovative techniques. Oxford Nanopore, on the other hand, has a distinctive way of identifying single molecules by using a nanopore in a possibly unique way. These nanopores can be synthesized with both the synthetic materials, like silicon and graphene, or with biological materials, such as proteins which form pores in the membranes, like alpha-hemolysin. Besides natural substances, such as pore-forming proteins, these pores also include synthetic materials (Clarke et al., 2009). Similar to the ones before, the labeling which detects changes in pH or the nucleotide base which it incorporated indicated, nanopores contrary work. It works by analyzing the DNA directly and there is no need for identifying the bases (Fig. 2.6).

Specifically, this approach manifests a way that the generated ionic current can be regulated and a DNA strand fully encapsulates the slip of the nanopore (Pugh, 2023). Some such molecular properties as size, diameter and so on can be explored from the trial shifts that signal produces. At the beginning, nanopore gate is exposed to some particular values of ionic current which is passing through it. As the distinct nucleotides have resistance properties, they hence breakdown the transmission of current in varied time frames. These timing interferes to know what molecule it is and how they are ordered so called alternations. Development of nanopore-based DNA sequencing approach seems to be the next step in the direction of launching

high-speed DNA sequencing method with the help of this kind of technology (Astier, Braha, & Bayley, 2006; Stoddart, Heron, Mikhailova, Maglia, & Bayley, 2009).

DNA strands are able to enter the nanometers thinly placed pores, which plays a key role in extracting DNA sequences by discrimination and with great proficiency. Likewise, PacBio highlights the technology which allows monitoring the work of DNA polymerase in real-time. This gives a possibility of obtaining dynamic information about the process, namely, about the DNA synthesis. What sets direct observation apart during DNA polymerization is its ability to directly collect long-read sequences, minimizing the necessity for PCR amplification or any other form of fragmentation (Jain, Olsen, Paten, & Akeson, 2016b; Rhoads & Au, 2015).

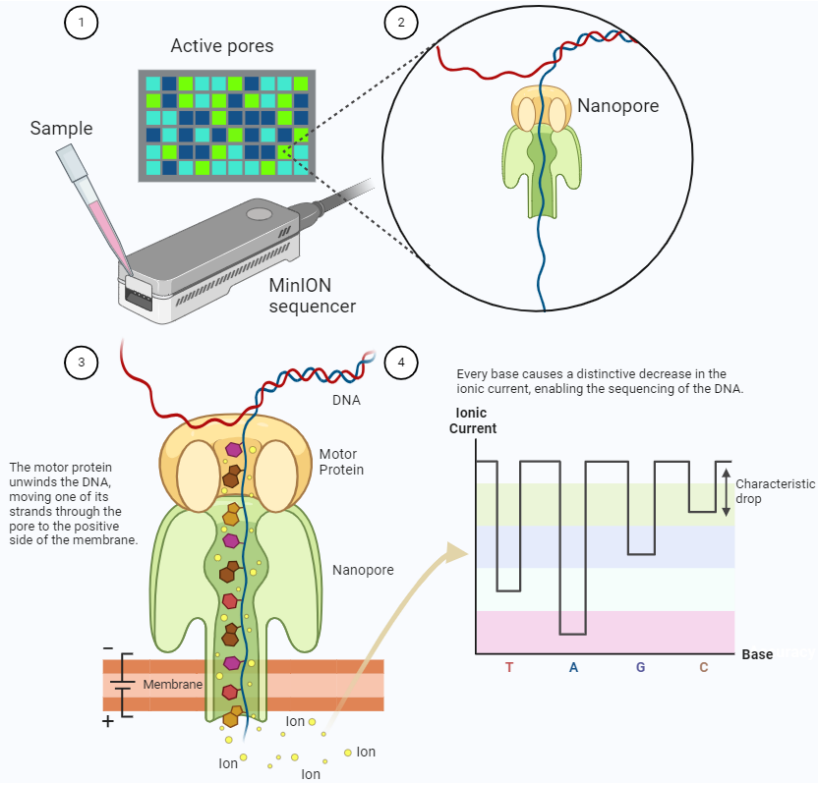

Fig. 2.6 Nanopore DNA Sequencing

These innovative technologies develop various intricate models which address shortcomings of the traditional sequencing techniques. They provide a number of research gateways into the complex genetic structures prompted by the types of structural variations they resolve. TGS technologies provide sequencing techniques that are direct, long-read and especially go beyond the boundaries of short-read sequencing approaches. This is why researchers are able to scrutinize both small and large genomic areas by employing an outstanding precision and accuracy (Venkata

Subbaiah, Hedaya, Wu, Jiang, & Yao, 2019). TGS technologies are going beyond the boundaries of traditional sequencing approaches to bring something new and entirely different. By their ability to conduct thousands of more experiments per generation and in live time, these technologies boost genomics research up to novel TGS level. Their range is manifold with regard to usage in different disciplines, such as deciphering complex genomic landscapes in detail and pinpointing structural variations along with being a milestone in reconsidering the genomic analysis and changing the concept of biomedical research.

2.2.4 Comparative Analysis, Implications and Methodological Advancements

Carefully comparing the evolutionary tracks such sequencing technologies pursue reveals a clear upward pattern and striking improvement in every key metric: speed, accuracy, scalability, and affordability. Meanwhile, each methodology has added something different to the complex tapestry of genomic research, and it is clear that there is an evolutionary trajectory where quality technological capabilities are being upended (McCombie, McPherson, & Mardis, 2019). The Sanger Method holds a revered position as the foundation of genome analysis, marking the inception of our understanding of the genetic code. While its methodical approach paved the way for a forthcoming revolution, it was hindered by labor-intensive processes and limited scalability. The subsequent evolution of Next-Generation Sequencing (NGS) or third-generation sequencing (TGS) technologies marked a significant paradigm shift, propelling genomics research into the high-throughput realm in the current era dominated by data-intensive genomic analysis (Mardis, 2008a).

Sanger Sequencing which is the earliest used method to Next-Generation Sequencing (NGS) and Third-Generation Sequencing (TGS) represent a remarkable journey which has evolved over the few years. Through the ages, these discoveries have offered vital tools for scientific exploration, from their use in a wide range of scientific investigations to their use across many scientific fields. These new instruments, though have not only affected the rate of scientific research but, also have become the platform in which genetic analysis in general has achieved a considerable expansion. The knowledge of these strategies will help in the interpretation of the Genetic codes which is rather complex. They have eminent ways of diagnosing diseases with high degree of accuracy in pathological laboratories, also being a boon in evolutionary biology, palaeontology and other subjects(Shendure & Ji, 2008). Although sequencing methods of nowadays evolved from the very beginning of the modern era driven by the unyielding demand for further development, technology remains the primary driving force of these advancements. This primitive work is the foundation, which is intended for science integration with medicine leading to

discover some cryptic messages of life in more special manner (Goodwin et al., 2016). Consequently, these innovations in the translational impact would include incremental elongation, toothpaste precision, speed, and a consistent decrease in the expense per base. The development causes a breakthrough in the field of genetics implying that it is a genesis of a new era in genetic researches and clinical diagnostics with an all-encompassing engage for growing medicine. These developments have had their radical presence in personal medicine, complicated genomic diseases of humans, and objective drug technique for each genotypes (Metzker, 2010). Due to their significant placing genetics they play a key role in other scientific areas. Today, time resolution that was once difficult can now practically be attained in clinical diagnosis and biomedical research, and these fields are being much more profoundly changed so that new synergy never before seen in the world is coming into existence (Table 2.1).

Table 2.1 Comparison of famous sequencing methods.

Method	**Sanger** (Sequencing Chain Termination)	**Illumina** (Sequencing by Synthesis)	**Ion Torrent Sequencing** (Ion emicon-ductor)	**Pacific-Bio** (Single molecule sequencing)
Read Length	400 to 900 bp[a]	50 to 250 bp	200 bp	3,000 pb
Reads[b]	-	Up to 3 billion	Up to 5 billion	36-76 thousand
Accuracy	99.8%	97,8%	97.8%	98.9%
Time	20 minutes to 3 hours	1 to 9 days	2 hours	30 minutes to 2 hours
Cost[c]	$2,500	$0.06 to $0.16	$1	$2
Pros	Wide application, Long individual reads	Sequence yield depending on equipment model	Less expensive equipment and fast	Longest read length and fast
Cons	Expensive equip-ment, unsuitable for great projects	Very Expensive equipment	Homopolymer Erros	Expensive equip-ment, low yield

[a]base pair [b]per run, [c]per 1 million $

2.3 Applications in molecular diagnostics

2.3.1 Disease diagnosis and prognosis

Sequencing of nucleic acid is the key technology that is applied to diagnose various diseases as well as to predict their genesis. This capacity to reveal one's genomic makeup in detail or target specific areas upon a researcher's request is the most significant characteristic of these technologies. This ability has resulted in a long way of understanding the molecular sources of genetic disorders (Beltran et al., 2015). Such technologies produce a massive amount of data which is helps professionals get insights into genetic variations. Such profound coverage with even more precision in detection and distinction of different kinds of medical problems, ranging from heredity diseases to cancers, infectious diseases, and rare genetic disorders, is now possible through this kind of genetic testing. These techniques which are advancing at an unprecedented speed are partly substituting the practices that have been in the mainstream medicine (Yngvadottir, Macarthur, Jin, & Tyler-Smith, 2009). Through the looking of sequence information, researchers can have the basic genetic data of the diseases, and then according to their genetic profiles, they can be denomiated. This is subsequently followed by various individualized targeted treatments, benefiting from the detailed patient's genetic makeup. The role of personalized medicine is critical in the current healthcare system and can contribute to better treatment outcomes, lessen adverse effects, and boost overall patient care satisfaction. While technology keeps improving further, the relative of personalized medicine is great and will not only have a bearing on patients but also the evolution of healthcare (Flusberg et al., 2010).

In terms of molecular diagnostics, the advent of nucleic acid sequencing seems to hold immense potential for healthcare revolution. It lays a foundation of such tomorrow wherein personalized medicine becomes an integral element of medical field not only depicting the great synergy between science and clinic but also the prominent role of scientific innovation. Such integration of these techniques takes traditional diagnostic methods to a greater height. Not only that, it massively reshapes the future with unique and peculiar care provision that is quite rich and essential. Through this activity, the core principle on care delivery is being changed, which is bringing the treatment era where treatment is prescribed based on one's genetic profile. This leads to improved outcomes and better patient care (Mardis, 2011).

2.3.2 Personalized Medicine and Targeted Therapies

Indeed, advanced sequencing technologies hold tremendous promise in uncovering detailed information about individual genetic variants, paving the way for personalized medicine approaches. For the first time in medical history, physicians can tailor treatments to the unique genetic makeup of each patient, increasing the likelihood of treatment success. The intricate genetic variations revealed by sequencing data drive the development of targeted therapeutics. These findings provide unprecedented specificity in treatment approaches (Collins & Varmus, 2015). Precision oncology serves as a prime example, where sequencing data are utilized to identify actionable mutations specific to certain cancers. This shift towards a precision-based approach allows for the conceptualization and implementation of targeted therapeutics for cancer, departing from traditional methods to a more precise and effective treatment approach (Woerner, Gallagher, Vockley, & Adhikari, 2021).

It transcends the one-size-fits-all paradigm for integrating technologies in personalized medicine in an era whereby therapeutic strategies are tailor-made with a match of individual genetic signatures of patients. One study showed that this individualistic approach leads to improved minimal side effects and therapeutic efficacy, heralding significant milestones that would be realized via amelioration of patient outcomes as well as quality of life (Petersen, Fredrich, Hoeppner, Ellinghaus, & Franke, 2017). The design of therapeutic interventions guided by sequencing insights heralds a quantum leap in medicine. This aligns genetic profiling with individualized therapeutic interventions, giving way to an era where health interventions are foreseen and fine-tuned against an individual's unique genetic blueprint. The possibilities of such potential, to expound in the light of sequencing technologies and emerging forms of personalized medicine, only such opportunities in patient care would lead to further individualized medicine, this time profound in its efficacy and precision (Wooden, Goossens, Hoshida, & Friedman, 2017).

2.3.3 Advances in Genomic Medicine and Therapy

The easy assimilation of sequenced data into clinical practice is merely one of the transformative facets of genomic medicine that have emanated from the interface linking genetics and the provision of health. It is one of the precision moves in genomic medicine; it uses extensive genetic data to establish fine details indicating how a patient will react to a prescription. The strategy is based on developing new dimensions of intervention where meticulous attention is paid so that minimal risks are ideally avoided for adverse reactions and, after that, to enhance pharmacological effects maximally realized via meticulously tailored treatments (Caudle et al., 2014). Genetic profiling has to do with the invaluable information on individual differences in pharmacologic efficacy, metabolism, and susceptibility to pharmacogenomics.

This large mass of genetic information is what pharmacogenomics uses to specify the genetic signatures behind the medication reactions. It allows a determination that brings customization into treatment plans and ensures the most beneficial selection and dose of medicine, specifically based on the patient's genetic composition. It helps increase treatment efficiency and reduces the incidence of adverse drug reactions, indirectly adding to patient safety and overall treatment outcomes (Oka, Ye, & Zuker, 2015).

Finding the genetic variations related to disease becomes one of the driving forces in developing new therapeutics and pharmacological targets. It is worth mentioning that genetic impairments play a core role in the invention of new treatments or the designation of pharmaceutical goals (Warburton & Sebra, 2023). The description of the gene-specific markers on the disease seems to have gained knowledge whereas serving as the guiding stars to a new field in therapeutic intervention. By focusing research on the field of single-specific drug production, the technology of this transformation serves the aim that the final product is capable of producing a desired drug that matches the change of genetic alterations that are related to major diseases. When the data obtained from clinical sequencing is fully integrated and merged with genetic medicine, it will create a disruptive model for the healthcare field. Genomic medicine, supported by sequencing technologies that fuel and define its momentum, could disrupt the paradigms in therapeutics, arming healthcare with highly predictive, finely tailored interventions truly impactful in meeting patient needs (Katsanis & Katsanis, 2013).

2.3.4 Recent Trends in Clinical Applications

A new paradigm of sequencing technologies is thus prompting the constellation of new trends that define clinical application beyond the domains of diagnostics of diseases and their tailored therapeutic options (Yin, Butler, & Zhang, 2021). Exact and expanded prenatal genetic testing shows the revolutionary potential of this approach for reproductive health. Prenatal genetic testing has become an area in which the early recognition of genetic abnormalities affords insight into fetal health that was previously unimaginable, thereby aiding both patients and healthcare providers. It is through consolidating these new developments (Gil, Accurti, Santacruz, Plana, & Nicolaides, 2017).

The second innovation in prenatal treatments is the new advent of non-invasive prenatal screening. These technologies for screening now allow the complete study of the fetus's circulating DNA with the mother's blood. It is for the first time that the inherent element of eroneous application pregnancy-related diagnostic techniques is in action. It is an emblem of hope through which expectant mothers will obtain inestimable early lessons in maternal care with maximum fidelity to the fetus and with no danger (Naccache et al., 2013).

It is worth noting that along with the sequencing technologies, cancer diagnosis and treatment have also been changed (Zhang et al., 2022). Clinical applications of the technology related to this field can be observed in the form of sequencing of group of genes, which are traditionally known as gene panels (Tung et al., 2015). This revolutionary technology using molecular sequencing technique provides genome-wide characterization of genes associated with multiple types of cancer resulting in a detailed "genetic portrait" of the molecular changes that lead to tumorigenesis (Kraus et al., 2017). The gene panel basically includes the key oncogenes, tumor suppressor genes, and genes related to the DNA repair ways to study within the range of the clinically relevant mutations. These genes, like TP53, BRCA1 and BRCA2, EGFR, KRAS, HER2 and so many others, are the principal components of carcinogenesis and therapeutic response (Aloraifi, Boland, Green, & Geraghty, 2015). Gene panels simultaneously investigate the expression of several genes with an unprecedented speed and accuracy within the confines of next and third-generation sequencers (Castera et al., 2014). During treatment, diagnostic panels including the Foundation One® Panel or the Oncomine™ Comprehensive Panel examines a number of genes, which are relevant in various cancer types from a medical practitioners perspective (Ali et al., 2023). Hence, the diagnostic process becomes much faster and the health care professionals can ultimately decipher the complex molecular networks which drive the abnormal development of cancer.

Moreover, the advent of metagenomic sequencing makes a major hurdle in the detection of microbial pathogens for diagnostic use in infectious diseases. Nonetheless, microbiota provides a new impetus to have metagenomic sequencing applied in microbial pathogen detection with prospect of taking the next step in pathogen identification and diagnosis (Adamopoulos et al., 2024). In addition, regarding an outline of the importance it gives to microbial community profiling, it is possible to diagnose and characterize diseases with almost 100% accuracy in all environments (Stranneheim & Lundeberg, 2012). In microbiological environments where matters are complicated, metagenomic sequencing has appeared as the fundamental approach for rapid and accurate diagnosis of infectious diseases. Its applications involve epidemiological research, outbreak investigations, and emerging pathogen surveillance; these offer critical information to healthcare workers required to counter the spread of infectious diseases (Almeida et al., 2019). As this dynamism expands through its present trajectory, these applications stand like a beacon toward an envisioned future for predictive, personalized, and most profoundly influential healthcare engagements in fulfilling the many challenges faced in clinics (Fig. 2.7).

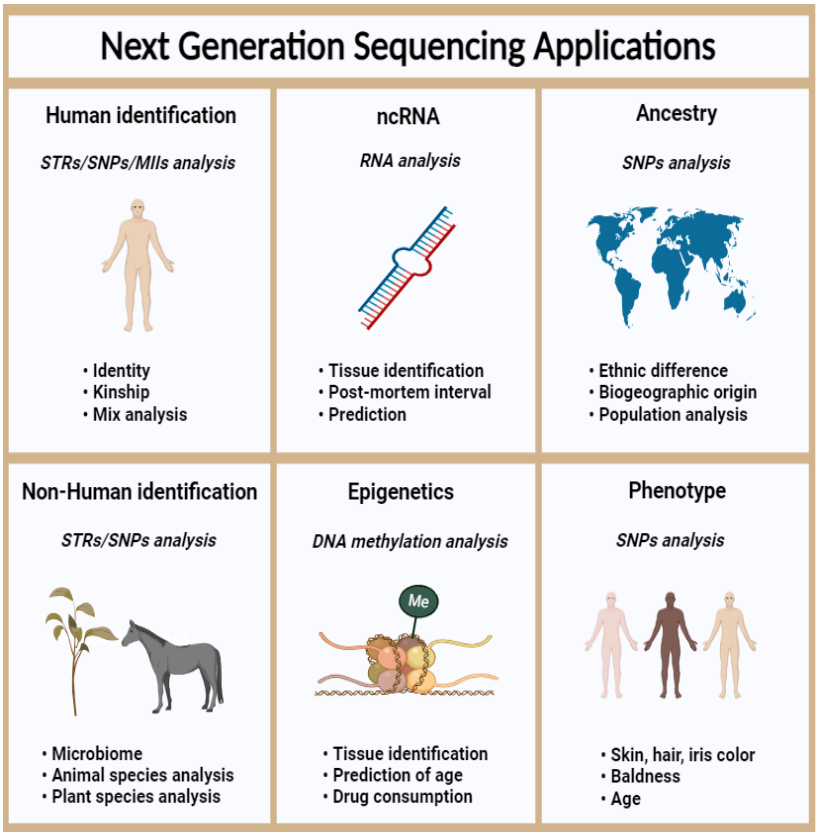

Fig. 2.7 Next generation Sequencing Applications

2.4 Challenges and Future Perspectives

2.4.1 Addressing Data Complexity & Challenges

The integration of sequencing data in molecular diagnostics is heralding a transformative shift to precision healthcare, reshaping the new contours of clinical practice. Justifiably, the enhancement of its accessibility and a clear improvement in the cost-effectiveness of sequencing technologies are signaling that a more novel era is coming wherein integrating such advanced methodologies may revolutionize patient care across several medical scenarios (Senter & Sievers, 2012).

The increasing availability and dropping costs heralds a quantum leap for healthcare, putting us closer to the day to one in which early detection of disease is not only a hope but becomes a reality (van Dijk, Auger, Jaszczyszyn, & Thermes, 2014). Incorporating modern advanced diagnosis into practice with conventional

medical science opens the prospect door wide for early disease detection with keen insight into the genetic structures. The accuracy in sequence data today allows this new method for handling health care to offer a revolutionarily proactive opportunity to intervene at early enough stages, which may translate to disease outcome changes and the betterment of patients' lives (Kalia et al., 2017).

These requirements provide a window for treatment specifics that resonate entirely with the need for completeness and the uniqueness these patients portray in their genetic signature (Nakagawa & Fujita, 2018). This wealth of data generated by such a sequencing analysis provides the way forward for medical professionals to select individualized treatments based on the genetic constitution of their patients. Therefore, this precision medicine approach will lead to an overreliance on precisely calibrated medicine through boosted efficacy, curtailed side effects, and departure from the one-size-fits-all therapy model. This integration positively affected different medical ranches and opened a gate to building a future when acute health care is available. (Kalia et al., 2017).

2.4.2 Circumventing the Bounds of Technology and Cost

Irrespective of how much effort has being put on a majority of the existing sequencing technologies to deliver results that are accurate and detailed, they are still faced with some obstructions that have limited the scope of discovery (Dorado, Galvez, Rosales, Vasquez, & Hernandez, 2021). While the technology has advanced tremendously over the years, there are still difficulties associated with defining repetitive regions and complex genomic landscapes accurately. The places where these are performed are intrinsically complex and prone to errors, which results in difficulties to show sequencing accuracy and faithfulness. The repetitive part and the complex genomic regions with their complicated nature cause serious difficulty due to their intricacy. Current sequencing tools usually drip with errors or gaps when addressing these regions. To overcome this issue, new sequencing approaches, advanced algorithmic methods and powerful bioinformatics tools have been invented. Such tools strive to boost the accuracy and reliability of sequencing, in this way helping researchers to escape the pitfalls of these complicated structural impediments (Callaway, 2020).

Furthermore, the significant price reductions that sequencing technologies have undergone suggest that increasing access, especially within resource-constrained healthcare systems, is currently the sine qua non toward universal uptake (Yohe & Thyagarajan, 2017). However, currently, this reduces the expense and makes it democratic for global health scenarios. This reduces the economic barriers associated with sequencing technologies through a need for constant innovation in design, cost of reagents, and even more efficient workflows that minimize costs associated with sequencing without any loss in quality or accuracy (Treangen & Salzberg, 2011). Attempts to change technological deficits and cut down expenses mark an essential

moment in shaping the future direction of development in sequencing technologies.

Today, the field of sequencing technologies operates at the intersection of innovation, technology, computational expertise, and strategic cost reduction. Accurate alignment of genome sequences, which includes complex ones, will safeguard the field of sequencing technologies. That means it will be cost-effective, globally accessible, and the basis of change in the global healthcare system. Meanwhile, superimposing the challenges that field is striving to overcome, a perspective of the sequencing technologies revolutionizing health care and biomedical research is still attractive. This will create new opportunities for accurate and autonomous patient care along with scientific experiments, and these defining moments will lay the foundation to a fresh perspective in healthcare and research (Seyednasrollah, Laiho, & Elo, 2015).

2.4.3 Ethical and Regulatory Implications

Central to these ethical deliberations is the paramount concern of privacy in genomic data. Paramount in these ethical considerations is the issue of privacy as far as genomic data is concerned. Therefore, appropriate regulatory policies must be applied because genetic material can be extremely complex (Masson et al., 2006). This necessitates strict protocols for managing, storing, and distributing genomic data to avoid avoidable violations through abuses, illegal access, or other breaches that could jeopardize personal privacy. However, one is still a highly controversial problem, which urges legal and ethical frameworks to protect public access to scientific research and their simultaneous right to privacy while promoting the former over the latter (Stansfeld et al., 2018).

Concurrently, informed consent emerges as a cornerstone in ethical genomic practices. Obtaining transparent, comprehensive consent that elucidates the implications of genomic analyses, including potential findings unrelated to the primary purpose, is a moral imperative. In their place, an element that becomes of fundamental importance for genomic practice entails that of informed consent. This requires complete, understandable, and explicit consent to explain in detail what may accompany genomic analysis, particularly the findings that are peripheral to the main aim. Understanding this kind of analysis's scope, risks, and benefits empowers persons (L. M. Johnson et al., 2017). It helps them choose where to invest their genetic info to promote self-determination and respect for preferences. There is a need for robust regulatory frameworks to handle the complex junction of innovative ideas brought onto the table and ensure that such implementations have a firm basis in ethics. They call for frameworks balancing an appeal for innovation, guaranteeing open, moral uses of genomic data in medical treatment. All these become the need to develop comprehensive laws based on ethical norms and interdisciplinary cooperation for supporting ethical quandaries and encouraging a sense of responsible use. This

approach also helps foster public confidence in the healthcare procedures and moral conduct of genomic research (McGuire & Beskow, 2010).

As far as human embryology and sequencing technologies are concerned, bioethics play a key role to ethically consider and ethically frame the handling and analysis of fetal material, mainly on IVF context (Ventura-Junca et al., 2015). Combining the rapid advancement in technology with the exquisite sanctity of human fetal development gives rise to deep moral dilemmas concerning the rights and dignity of a fetus and wider social effects for the humanity. From the center of the discussion are the possible wrongdoings that can be done with embryonic material such as the ethical concerns of creating designer babies through germline genome editing in IVF or embryo processing (Harper & Schatten, 2019). Apart from this, consent related issues, privacy concerns and responsible utilization of genetic information were also huge, especially in the context of prenatal genetic testing and screening. Balancing scientific development with medical ethics necessitates a vital regulatory and oversight system to make certain that research practice and clinical methods comply with the principles of beneficence, autonomy, and justice (Burgess, 1994). Moreover, facilitating exchange of ideas and interacting among scientists, policymakers and the public is very valuable to traverse the complicated ethics of human embryology. Via the mentioned dialogues we will be able to create conditions for the existence of a long-term guarantee of well-being (Anifandis et al., 2022).

Genomic data in healthcare is undergone a remarkable period of time, and the ethical issues have been transformed using the technology as an instrument. The continuing advances in the field of genetics and medical genetics serve as proof of the necessity of the faithful communication, ethical assessment, and cooperation among the genetic research and healthcare providers. Governments, international organizations and industries have a duty to promote accessibility by engaging in the open dialogue on the potential concerns that all of us might have, and shaping the future where genetic technologies will be good tools to drive healthcare forward, although not at the cost of individuals' rights, privacy and dignity.

2.4.4 Future Prospects and Technological Innovations

In the segments of sequencing technologies, certain developments that are in the offing are by far glorious of what these technologies can be accomplished. In my view that the future advances in molecular imaging will be in the field of changing world from high resolution, continuous visualization and complete understanding of biological systems. Of these, three stand out as noteworthy: in the following areas – the low costing and the personalized genomics for each patient, illustrate the tech world can be altered as well by the Safe sequencing technology(Marx, 2021b).

From that moment on, single-cell sequencing will be a guiding light that will continue to lead this discipline’s journey through the future and it is going to show what

the path of this field's evolution will be like. This approach will lead the scientific community to the next step, that of fourth (4th) generation sequencing. The fourth generation of sequencing technology opens up prospects for transcriptomic analysis in tissue or cell level as far as malignancy treatment is concerned. It may be possible to establish a standard method for the sequencing of tissue or cell samples if technological challenges be solved by advancing technological advancements. Under the wide sky of this landbreaking feat, scientists are then equipped with the ability to scrutinize individual cells thereby revealing the intricacies of biological processes at a cellular level. Single-cell sequencing leads to unmatched resolution, where researchers are given the chance to uncover more about cellular connections, development cycle patterns and the diversity of the single cells within tissues and biological systems. Lastly, researchers are able to map out cellular lineages and follow temporal development of cells with this technique. Furthermore, single cell sequencing innovates on traditional bulk sequencing approaches which are the only ways to explore diminutive genomic variations within each individual cells states. This provides a basis to address biological problems that were too delicate and of too high a technicality to unravel before. Single-cell sequencing provides a highly effective means of gaining fresh perspectives in cellular biology and a revolution is imminent as we become to know complex biological systems (Marx, 2021a).

Long-read sequencing technologies represent a paradigm shift in general genomic analysis of genomes. Long-read sequences, provided by recently conceived long-read technologies, have been proposed to conquer the constricting features of short-read ones, enabling the tracing of intricate patterns of genomic architectures with unprecedented fidelity. Thus, long-read technologies bring even closer to a future where the discrimination of genomic features will become a simple task through increased structural variant, repeat region, and complex genomic landscape study (Rhoads & Au, 2015).

The involvement of a whole variety of 'omics' disciplines is crucial because scientists should decipher the complex relationship between the genetic variants, protein levels, and metabolite profiles in order to make a coherent sense of the system. Researching this issue by utilizing these integrating approaches brings the opportunity not only to see various processes that these biological systems go through but also to comprehend how these processes work simultaneously. Thus holistic method provides a comprehensive analysis of how the genetic changes will have the impact on proteins production and on the metabolite profiles what in future will give insight on the complex functioning of biological systems (Worheide, Krumsiek, Kastenmuller, & Arnold, 2021). With this novel systematic skill, new genomic assessments are possible for understanding and applying in the future. And merging them change them to one super tool that endows you with features that are jaw dropping, complex and detailed. This integration will be the main driver of science's frontiers changes, a drug discovery breakthrough, better healthcare interventions, and generate fundamental life systems understanding. Ultimately, the emergence of cutting-edge innovations gives rise to the most exciting era of scientific discovery ever. They

encompass an exactness and a Greek of the understanding that lately has not been conceivable, and it will propel us towards amazing discoveries that will be very crucial in the mending of diseases and even in the research of anatomy (Buitink et al., 2016).

2.4.5 Precision Medicine and Healthcare Transformation

Precision medicine symbolizes the signal of a new age in health care initiated by the super-integration of technologies for sequencing in routine clinical practice (Hynes, Pang, James, Maxwell, & Salto-Tellez, 2017). The approach turns out to be a radical turn toward tailored health care approaches, which are precisely adjusted to the individual genetic makeup of the patient. The basics of this are grounded in deep insights from individual genetic profiles. Such integrations will help promote proactive, predictive, personalized care and pave the way for removing vestiges of generic health (Strianese et al., 2020).

To this extent, precision medicine ensures that development will occur concerning the personalization of treatment protocols in precise consonance with the individual genetic constitution of each patient (Sisodiya, 2021). All this is made possible with a massive amount of data made available to healthcare professionals in sequencing analysis to interpret their patients' complex genetic profiles and devise treatment strategies that benefit the patients while avoiding significant risks. It would, therefore, be an area under which much could be covered in areas of health disciplines: patient management, therapeutic interventions, predictive diagnostics, and disease prevention and diagnosis—all of which, especially the latter, are the integral features on which precision medicine anchors. The genetic data contain predictive potential of illness predispositions with early identification experience. It makes it possible for medical care experts to provide prevention or treatment when onsets are embryonic. Furthermore, this proactive approach, instead of a reactive one, encourages changing the course of diseases and improving some health outcomes (Qoronfleh, Chouchane, Mifsud, Al Emadi, & Ismail, 2020).

This precision medicine revolution changes the underpinnings of healthcare at its very core. With this change, the scientific community focuses on early detection, personalized treatment approaches, and prevention. As a result, it would increasingly move towards a patient centric, predictive, and curative healthcare system with time as sequencing technologies advance while getting fully integrated into practice(Sisodiya, 2021). This shift in thinking toward precision medicine consists evidence of the evolutionary potential of sequencing technologies in creating a predictive and personalized healthcare environment that offers patients unmatched advantages and ushers in an era of exceptional healthcare(K. B. Johnson et al., 2021).

2.4.6 Collaboration and Transdisciplinary Investigations

Overcoming the complicated problems associated with nucleic acid sequencing requires a cohesive, inter-institutional, and interactive effort. Realizing its maximum potential requires a concerted integration of knowledge from varied fields like clinical medicine, computational biology, genetics, and policy-making. Such cooperative synergy, followed by interdisciplinary research, is an intrinsic criterion indispensable for cutting-edge advances in molecular diagnostics and overcoming obstacles in this domain (Morey et al., 2013). This joint effort has skills at its base arising from integrating computational biology with genomics. A junction point of all these areas stimulates the appearance of sophisticated methods and bioinformatics tools in genome sequencing (Kumar et al., 2024).

Actual translation of the above sequencing technology advances to real-world clinical applications, on the other hand, critically depends on integration with clinical expertise into the context mentioned above. Clinical medicine supplements cutting-edge research by providing direct patient care experience, knowledge of practical healthcare applications, and knowledge of disease pathophysiology. Integration of these three facets ensures that the technology improvement that develops is translated into clinically applicable sequencing applications (Stranneheim et al., 2021). Hence, ethics and regulatory frameworks will be a critical aspect and play a heavy role in the formulation of spheres for the purpose of determining how genomic data will be used ethically and its ethical deployment and use. The urgency for the strong policies, where the ethics of the sequence and technology together is used to ensure an equitable distribution and utilization is the talk of the hour (Amoakoh-Coleman, Vieira, & Abugri, 2023).

Their triumph was not only won by overcoming obstacles in processing the nucleic acids but also through commitment to use its full potential too. Fusing together these multiple in discerning enterprises with determined efforts towards integrated efforts, they are driving to untiringly invent advanced systems for the molecular diagnosis of diseases. Genetic technologies can interfere with the topics and the directions of science and health, fundamentally altering the physiognomy of healthcare and biomedicine (J. C. Liu et al., 2022). Hence, this domain evidences that via multinational collaboration and harmonization of expertise the two poles of the sphere, specialists and generalists, might embrace the comprehensive and holistic principle of environmental stewardship and sustainability. Their approach shall gain the appreciation of academia, business, consumers, and policy. This scope of synergy may represent such change since it is an evidence that this form of transformation in science would have not been possible without the power of this interdisciplinary approach latter on as a fuel for this transformation (Alshangiti, El-Damhougy, Zaher, Madani, & Mohamady Ghobashy, 2023).

2.5 Summary

The historical trends of evolutionary course in Nucleic acid sequencing methods show a probable symbolization of scientific excellence. It accompanies us in our journey to rediscovering and liberating the key player in genomics and molecular diagnostics. Through the lens of history, one can see that every milestone from the beginning of the Sanger method to the Big Data era of the Massively Parallel Sequencing (MPS) has always been a celebration of the human energy and undying progress, fervently advancing unknown territories and bringing sea-change in the landscape of biomedical research and clinical practice. This was, however, a very elaborate but time-consuming procedure; yet, at the same time, it unlocked the blueprint of genetic material, allowing abundant new knowledge in the realm of genetic landscapes and embryonic molecular diagnosis. The new method, Massively Parallel Sequencing (MPS) changed the scene drastically into a complex technological strength characterized by speed, capacity, and cost efficiency of unprecedented levels. This evolutionary leap is moving genomic analysis an order of magnitude beyond anything that has come before, effectively opening the way for a realm of high-throughput, data-intensive sequencing methodologies (Gao & Smith, 2020). It is a tale woven through the mists of time from which nucleic acid sequencing has evolved so much more than the history of technological development; it is the story of persistence in creativity, strength in adversity, and single-minded commitment to an unswerving quest for unravelling the secrets of the human genome. Ultimately, as we reach the end of this thrilling encounter, the discovery of nucleic acid early sequencing becomes an indispensable milestone on the road of sciences. They are not only used in the nuclear medicine to develop individual treatment protocols, but they can also be applied to different fields which are fancied by breaking out sensational new records. The preceding critical inquiry has demonstrated that contemporary changes within genomics, from time-intensive and step-wise methods, are moving towards those that demonstrate high throughput and parallel capacity. This evolutionary journey will represent both an ode to the advances won by scientific acumen and a heralding one of nothing less than the advancements to come.

The passage of nuclear DNA sequencing technologies from the tedious, gene-by-gene system to the incredibly quick parallel system represents a crucial page in the history of science. Applications in personalized medicine, diagnosis, and therapeutics, which fall under the nucleic acid sequencing field are key for making it a primary component of a future precision healthcare. Beyond compare, the flexibility and dynamism offered by this high-throughput, parallelized sequencing approaches have entirely revolutionized the scale and perception of genetic study, with several hitherto unimaginable insights now available to researchers and doctors regarding the intricacies of the human genome. These methods work quickly and lightly without a hitch. The paradigm is changed in disease diagnosis and prognosis by nucleic acid sequencing diagnostics through their integration into precision health

interventions (van Galen et al., 2019). The accuracy and precision of this analysis will be able to identify the genetic aberration (such as mutations) underlying most diseases; therefore, it contributes to patterns of accurate diagnosis and prognostic detail in planning treatment options.

These progresses of personalized medicine are centered on the core belief that nucleic acid also serves as a chance to prevail. An up to date comprehension of a patient's underside genome for now will be the basis for individualized treatments, which whole genome sequencing may possibly accomplish. This target-specific treatment-based scheme means that in the future this information will be used to guide individualized medications based on each person's genetic printing. Such an interrelation between nucleic acid sequencing and health-related initiatives is manifested with no equal opportunities impacting the precision paradigm of healthcare. This transformative experience underscores the tremendous potential that sequencing technologies will play in leading a healthcare system that is unconventional and individual-focused, preventative and predictive, with a tremendous promise to advance medical science and individual health. Finally, it must be highlighted that this life-altering undertaking was filled with incessant hurdles that required sophisticated and original problem-solving techniques.

Sequencing of nucleic acids deals with numerous problems like data complexity and lack of technology, ethics, and finance. However, these challenges have been a source of the most creative breakthroughs, drawing the boundaries of science that were hopeless before. A major problem is the fact that sequencing technologies generate varieties of data that require the management of large genomic datasets. However, data complexity manifests truly in the tasks of detection of rare variants or structural variations amongst the large volume of data. While these problems can not be fully resolved, bioinformatic tools and computational methodologies already hold the power of innovation that can move beyond this complexity. These innovations target on cracking the mysteries of the genome at the highest level of accuracy and preciseness with the objective of moving the field forward (L. Liu et al., 2012).

Along with data complexity, restraints on technology are another major factor limiting the completion of genome analysis. The most pivotal disadvantage of the sequencing approach, namely accurate repeat abundance depiction and complex genomic procedure completion is the next step in the evolution of sequencing technology. Recently emerging sequencing techniques present the possibility of enhancing precision, standard, and affordability. This is the step where these technologies can facilitate more for a sustainable health care that can ensure greater effectiveness and accessibility. Despite this, the integration of sequencing technology into clinical practice is not free of ethical concerns. The legal frameworks should be robust enough to protect individuals and privacy, while at the same time guaranteeing the informed consent. The responsible use of genomic data requires a crucial realization: policies that emphasize the balance of flexibility with ethical principles are necessary. Among all the obstacles, there is yet a field of sparkling of opportunities for the developments of studies in genomics and healthcare (Lantos, 2019). The horizon

of sequencing technologies has within it the prospect of breakthroughs that will be able to tackle the current flaws.

Instead of disturbing, their complexity and demanding character should encourage our further advancement, which would serve as a revelation for the strength and inventiveness found within the scientific world. As a basic recognition in medicine of precision, therefore, the nucleic acid sequence acceptance into daily health practice is the call sign of heralding the change in the delivery of health to this new age. Such new paradigm shifts are enshrined in precision-guided intervention and rely on a robust foundation fortified by collaborative efforts and ethical fortitude, coupled with relentless technological innovations. The placement of nucleic acid sequencing within the the fabric of daily clinical practice imposes some overall watershed moment where healthcare begins to change toward an era when intervention is customized by being precision-centric. This transformation trajectory was shored up by integrated techno-advances in sequencing represent a shift from seismic ground toward paradigms of healthcare that must be personalized and predictive—never just preventive.

The history of nucleic acid sequencing, from pioneering through all breakthroughs to the most recent advanced techniques, is a story full of revolutionary shots over time and technological barriers. It points at something crucial in science, and, at the same time, it stands for a shift in our perception of how we should get to know this enigmatic detail in DNA molecules. This path is a journey through a series of attempts and efforts to improve everyday life; it contains the maximum of human perseverance as a positive factor and proves our inventive nature. By settling in the fundamental ground of the Sanger method in the early 80s and further encompassing HiSeq of MPS and beyond, each Subsequent experiment gets humans closer to a real understanding and implementing the practice with the knowledge involved. Using more precise terms to describe the development of such scientific areas that have nucleic acids implication is also almost immeasurable part of the progress in scientific disciplines. It is the image of being the of progress for humanity's horizons in terms of comprehension of genetic complexities as well as causes of diseases, which results in such that health care therapies can be tailored. (Maynard et al., 2020). The revolutionary impact is realized in different genres of revelations, like disclosing the genetic causes responsible for different illnesses and devising individualized treatment programs according to each patient's unique genetic constitution. The story that pertains to nucleic acid sequencing, however, extends much beyond science and incorporates a type of revolution in perception as to how information on genes is supposed to be understood and used in the first place. Its eternal history highlights the future with many possibilities in line with the waves of scientific change. The nucleic acid sequencing is a timeless monument of humanity as it goes forward during the beginning of this revolutionary age. It has exciting prospects for the future, where genetic information brought about by applying novel sequencing technologies accelerates medical advances and scientific breakthroughs even to levels of successes and progress not even imagined before.

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Chapter 3
Fats that Heal or Fats that Kill: The Effects of Specific Omega-3 and Omega-6 Polyunsaturated Fatty Acids on Cardiovascular Diseases

Stephanos Christodoulides, Charalambos Michaeloudes, Anastasis Stephanou and Ioannis Patrikios

Cite this chapter:

Christodoulides, S., Michaeloudes, C., Stephanou, A. and Patrikios, I. (2025). Fats that Heal or Fats that Kill: The Effects of Specific Omega-3 and Omega-6 Polyunsaturated Fatty Acids on Cardiovascular Diseases. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 65-95). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 3
Fats that Heal or Fats that Kill: The Effects of Specific Omega-3 and Omega-6 Polyunsaturated Fatty Acids on Cardiovascular Diseases

Stephanos Christodoulides, Charalambos Michaeloudes, Anastasis Stephanou and Ioannis Patrikios

School of Medicine, European University Cyprus, Nicosia 2404, Cyprus.

Keywords: n-3/n-6 polyunsaturated fatty acids, atherosclerosis, molecular mechanisms, cardiovascular diseases, inflammation, epigenetics

Chapter Abstract: Supplementation with specific n-3 (omega-3) polyunsaturated fatty acids (PUFAs), primarily entailing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been shown to reduce key risk factors of atherosclerosis, the main cause of cardiovascular diseases. Specifically, n-3 PUFAs attenuate triglyceride levels, vascular inflammation and lipid oxidation. EPA and DHA exert their actions through common and differential molecular mechanisms that include changing the composition of cell membranes and plasma lipids towards a less inflammatory profile, through transcription factor activation, and even by affecting epigenetic mechanisms. On the other hand, a higher intake of specific n-6 (omega-6) PUFAs, arachidonic acid (AA) in particular, might increase cardiovascular disease risk because it is known to be involved in thrombosis and inflammatory pathways. However, increased levels of the 'essential' n-6 linoleic acid (LA) have been significantly correlated with lower risk of CVD. Indeed, LA has been shown to regulate molecular mechanisms involved in reducing low-density lipoprotein cholesterol. This chapter summarizes the current clinical and molecular evidence on the effect of n-3 and n-6 PUFA supplementation in atherosclerosis and the role of the n-6/n-3 PUFA ratio in the optimum response to supplementation.

3.1 Introduction

3.1.1 Cardiovascular Disease

Cardiovascular disease (CVD), comprising myocardial infarction and ischemic stroke, ranks as the leading cause of mortality worldwide. Atherosclerosis, characterized by the accumulation of lipids and fibrous deposits within major arteries, serves as the principal etiological factor of CVD (1). Stenosis induced by atherosclerotic plaque deposition, alongside thrombotic events secondary to plaque rupture might lead to blood flow restriction in crucial vessels resulting in myocardial infarction or ischemic stroke (2).

Myocardial infarction, precipitated by myocardial ischemia, entails myocardial necrosis and activation of cell death pathways triggered by oxygen deprivation (3). The primary pathological aetiology of myocardial infarctions is linked to coronary artery disease, characterized by obstructive lesions formed by the accumulation of atherosclerotic plaques within the coronary vasculature (4). Myocardial injury may progress to heart failure, characterized by structural and functional aberrations within the myocardium that limit ventricular filling or blood ejection, leading to inability to meet the demands of circulation (5). Ischemic stroke is characterized by compromised cerebral perfusion, affecting either part of, or all regions, of the brain, resulting in cerebral tissue injury and some degree of neurological impairment. The predominant etiological factor of ischemic stroke is attributed to atherosclerotic plaque rupture and subsequent thrombus formation within the carotid artery (6).

3.1.2 Atherosclerosis

Atherosclerosis primarily originates from lipid accumulation, notably low-density lipoprotein (LDL) and remnant lipoprotein particles, within focal arterial segments. It involves an inflammatory cascade, particularly at sites of disturbed non-laminar flow, such as artery branch-points. The predominant risk factors include elevated levels of LDL cholesterol (LDL-C), hypertension, diabetes mellitus, smoking, advancing age, and familial predisposition. Additionally, sedentary behaviour, obesity, low fibre intake, consumption of diets high in saturated and low in mono-unsaturated fat, and certain genetic anomalies may further increase the risk (7).

Atherosclerosis predominantly results from lipid entrapment within the intimal layer, facilitated by interactions with the extracellular matrix. This process induces modifications that drive chronic inflammation, particularly at susceptible sites within the arterial vasculature. This pivotal process, integral to all phases of atherogenic progression, commences with the formation of nascent fatty streaks within the arterial intima. Subsequent maturation leads to the development of fibrous plaques, ultimately evolving into rupture-prone atherosclerotic lesions (8, 9). At the molecular level, oxidation of LDL within the intima triggers pro-inflammatory

cascades, eliciting chemotactic signals that attract monocytes to the arterial wall. Upon arrival, these monocytes undergo differentiation into macrophages. Macrophages bind and internalize lipoproteins via LDL scavenger receptors, leading to the formation of foam cells, thereby contributing to the genesis of the atherosclerotic lesion. Concurrently, inflammatory mediators facilitate the recruitment of vascular smooth muscle cells, which synthesize extracellular matrix proteins, resulting in the development of a fibrous cap that encases the lesion, thus forming an atherosclerotic plaque (10). Decreased synthesis of extracellular matrix proteins by smooth muscle cells, coupled with increased matrix metalloproteinase secretion by foam cells, might result in fibrous cap thinning and eventual plaque rupture, leading to thrombotic events (11-13). In advanced atherosclerotic lesions, there is a notable increase in the incidence of smooth muscle cell and macrophage cell death, mediated by apoptotic or necroptotic mechanisms (14, 15). The inability of macrophages to effectively clear apoptotic and necrotic cells through efferocytosis initiates the formation of a necrotic core, thereby precipitating inflammatory responses and thinning of the fibrous cap (10, 16).

Therapeutic approaches aim to regress coronary atherosclerosis by using strategies geared towards delaying the progression and rupture of atherosclerotic plaques. Central to these efforts is the management of risk factors, including elevated LDL-C levels, hypertension, and diabetes, through interventions such as dietary modifications, exercise regimens, smoking cessation, and pharmacological interventions (17-19). Statins represent the cornerstone of therapeutic interventions for reducing LDL-C levels and preventing cardiovascular morbidity and mortality. Complementary to statins, a range of pharmacotherapeutic agents, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, beta-blockers, calcium channel blockers, and vasodilators, are employed in the management of hypertension (20-22).

3.1.3 Dietary Management of Atherosclerosis

Management of atherosclerosis may be facilitated through adherence to a dietary regimen characterized by high fibre content and enriched with mono- and poly-unsaturated fatty acids, while minimizing intake of saturated fatty acids (23). The n-3 and n-6 polyunsaturated fatty acids (PUFAs) have received significant interest due to their potential to influence critical CVD risk factors, including cholesterol and triglyceride levels, lipoprotein oxidation, vascular inflammation, and thrombogenesis. However, clinical investigations on the impact of n-3 and n-6-PUFAs on cardiovascular well-being present conflicting results, engendering ambiguity in their precise effect (24, 25). This review aims to comprehensively examine the current scientific literature regarding the influence of n-3 and n-6 PUFAs on CVD, with a specific focus on atherosclerosis.

3.2 Current Status of Knowledge

3.2.1 Biochemistry and Structural Morphology of n-3 and n-6 PUFAs

n-3 (omega-3) PUFAs constitute a group of elongated chain cis PUFAs. The designation "n-3" denotes the positioning of the initial double bond, located three carbon atoms away from the methyl terminal group (26). Alpha-linolenic acid (ALA; C18:3) serves as a precursor to the synthesis of longer chain (LC) n-3 polyunsaturated fatty acids, notably eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) (27). ALA is categorized as an 'essential' fatty acid, necessitating dietary intake due to its inability to be endogenously synthesized in the body. The primary dietary source of LC n-3 PUFAs is derived from oily fish. n-6 (omega-6) fatty acids represent the second pivotal family of PUFAs. Linoleic acid (LA; C18:2) serves as a precursor to the LC n-6 PUFA, arachidonic acid (AA; C20:4). Principal sources of dietary n-6 PUFAs include the common vegetable oils utilized in cooking, such as sunflower and soybean oil, along with foods derived from livestock and poultry (28).

It has been widely acknowledged that the present Western diet exhibits a notable ‘insufficiency’ in n-3 PUFAs, characterized by a ratio of n-6 to n-3 of 15-20:1. This ratio significantly surpasses both the optimal 4:1 ratio and the ideal 1:1 ratio, as recommended for a balanced n-6/n-3 nutritional intake (29). However, decreasing n-6 PUFA consumption is not obligatory to attain the optimal ratio. As per findings by Zhao et al., n-6 PUFA intake can be undertaken without eliciting detrimental effects, provided that adequate consumption of n-3 PUFAs is achieved. Furthermore, ensuring a satisfactory intake of n-6 PUFAs is pivotal for attenuating LDL-C levels, underscoring the significance of adequate intake of both n-3 and n-6 PUFAs in reducing CVD risk (30)

3.2.2 Clinical Evidence on the Effects of n-3 PUFAs on CVD

Epidemiological investigations have revealed a favourable association between the consumption of oily fish and beneficial cardiovascular outcomes (31). The evidence supporting the advantages of oily fish consumption appears to be more robust in secondary prevention compared to primary prevention settings (32). For example, researchers from the GISSI Prevenzione study (1999) demonstrated that dietary supplementation with LC n-3 PUFAs (1g/day) among individuals post-myocardial infarction led to a 21% reduction in overall mortality and a 45% decrease in sudden death incidences (33). Furthermore, a recent pooled analysis of four cohort studies revealed a correlation between the consumption of oily fish and reduced mortality risk in individuals with previous CVD (34). These advantages have been attributed to the presence of LC n-3 PUFAs, particularly EPA and DHA, predominantly found in oily fish (35). ALA typically exhibits lower efficacy in eliciting biological effects,

primarily due to its inefficient conversion rate (<5%) to EPA and DHA in humans (27). The inefficient conversion of ALA into the LC n-3 PUFAs is attributed in part to the high and rising levels of dietary n-6 PUFAs. The n-6 PUFAs compete with ALA for the same enzymes within the metabolic pathway, thereby hindering the conversion process (36, 37) (Fig. 3.1).

The beneficial impact of the LC n-3 PUFAs on decreasing the risk of cardiac mortality seems to stem from their integration into cardiomyocyte phospholipids, displacing AA (38, 39). The inclusion of n-3 PUFAs, notably EPA, within cellular membranes can lead to the synthesis of different eicosanoids, which may confer greater cardioprotective effects compared to those arising from the AA cascade (40-42). Several potential mechanisms underlying the cardioprotective properties of n-3 fatty acids have been proposed, with their impact on blood lipid profiles, specifically triglycerides (TGs), LDL and high-density lipoprotein (HDL), linked to the risk of atherosclerosis, being the most scientifically proven (43, 44). Notably, the most consistently-observed effect of n-3 PUFAs is the reduction in serum TG levels (45).

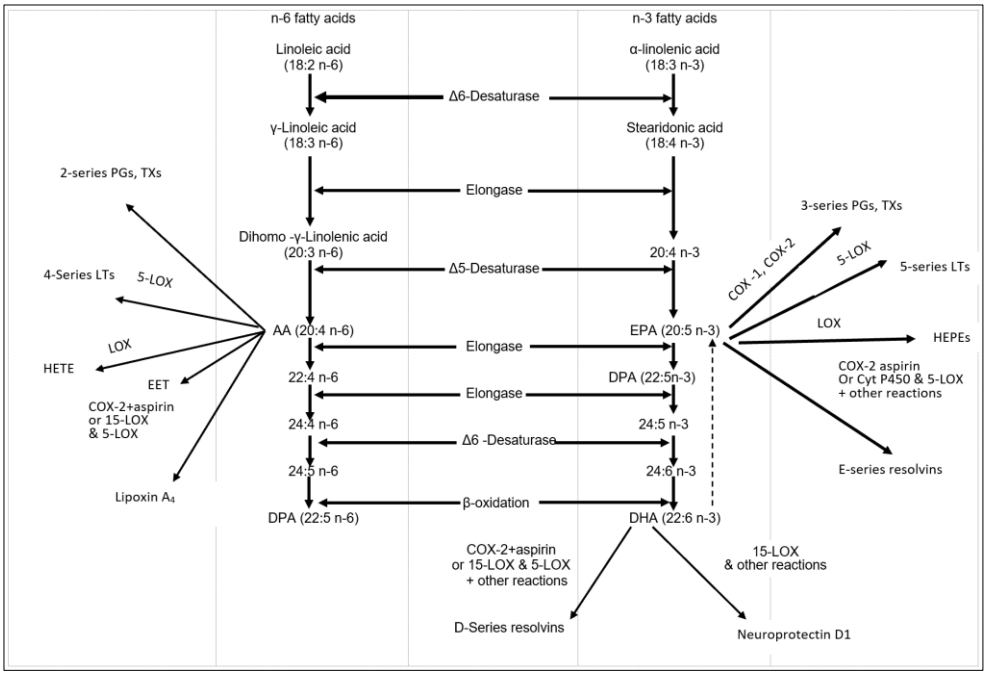

Fig. 3.1 Synthesis of n-6 and n-3 polyunsaturated fatty acids in humans. Outline of the formation of AA, EPA and DHA and the metabolic pathways for their conversion to eicosanoid (pro and/or anti-inflammatory) and other lipid mediators. Both linoleic acid and α-linolenic acid are elongated, desaturated and β-oxidised using the same enzyme system.

3.2.2.1 Effect on Triglycerides

According to Milte et al., elevated circulating TG levels represent an autonomous risk factor for CVD and exhibit correlations with both the progression and severity of atherosclerosis (46). In contrast, Torrejon et al. argued that while the involvement of LDL-C in CVD development is firmly established, the significance of circulating TG concentrations in this context remains a subject of debate (47). Nevertheless, as outlined by the British Nutrition Foundation (BNF) in 2005, there appears to be an indirect association between TGs and CVD risk. Studies indicate that decreasing TG levels results in decreased concentrations of small, dense LDL-C, consequently reducing the risk of CVD (48).

The most consistently observed impact of n-3 PUFAs is the reduction in both fasting and postprandial serum TG levels (47, 49). However, the precise dosage and duration of intervention required to achieve the optimal TG-lowering effect remain uncertain (46). To date, most of the studies have indicated a substantial decrease in TG levels (ranging from 25% to 30%) following treatment with doses equal to or exceeding 3g per day of LC n-3 PUFAs, primarily sourced from fish oil (50). Conversely, evidence suggests a much lower effect when lower doses are employed in the treatment (51). Across studies, duration varied from 6 to 104 weeks, yet the optimal intervention duration remains elusive. Notably, investigations failed to reveal any statistically significant difference in TG levels concerning longer-duration trials, exceeding 16 weeks, compared to those of shorter-duration lasting 16 weeks or less (31, 52).

In a double-blind, randomized, placebo-controlled parallel study, supplementation with DHA at a dosage of 3g per day for a period of 45 days resulted in a significant reduction in fasting TG levels among hyper-triglyceridemic men, with decreases ranging from 25% to 30% (53). In contrast, a double-blind, placebo-controlled study conducted by Yusof et al. (51), investigated the impact of a daily dose of LC n-3 PUFAs, comprising 1.8g of EPA and 0.3g of DHA, which closely mirrored the upper limit of daily dietary intake (2g of LC n-3 PUFAs) recommended by the Scientific Advisory Committee on Nutrition in the United Kingdom (54). However, this intervention demonstrated no significant effect on TG levels among normo-triglyceridemic individuals. Yusof et al. suggested that beyond the lower dosage administered, the observed absence of efficacy could potentially be attributed to the relatively poor content of DHA in the oil formulation used (51). Current understanding suggests that DHA may exhibit greater efficacy in reducing TG levels compared to EPA (55). Moreover, research indicates that the TG-lowering potential of DHA is higher in individuals with hyper-triglyceridemia than in those with normal TG levels (56, 57). A comprehensive meta-analysis encompassing 47 studies with a total of 16,511 participants examined the efficacy of n-3 PUFAs in managing hyperlipidemia. This analysis revealed a mean reduction of 14% in TG levels among hyper-triglyceridemic individuals following a 6-month treatment regimen, with an average daily intake of 3.25g of EPA and/or DHA (31). Nevertheless, it is

noteworthy that this dosage surpasses the safety threshold of 3g/day set by the U.S. Food and Drug Administration (FDA) (58).

3.2.2.2 Effect on Low-Density and High-Density Lipoprotein Particle Size

Studies have shown that LDL particles with smaller diameter and higher density, particularly the LDL-3 subfraction, exhibit higher susceptibility to oxidation. Furthermore, these smaller, denser LDL particles possess an enhanced capacity to permeate the intima compared to larger, less dense LDL particles like LDL-1 and LDL-2 subfractions, as shown by Harper et al. (59). Consequently, LDL particles with smaller diameter and higher density have been associated with an elevated risk of coronary heart disease (59). In a recent systematic review by Chary et al., the association between small dense LDL particles and increased CVD risk was concluded (60). Torrejon et al. reported a significant increase in particle size with DHA supplementation, indicating a potential benefit (47). Moreover, Kelley et al. showed that DHA supplementation leads to a reduction in plasma TGs, thereby decreasing the abundance of small, dense LDL particles and subsequently decreasing CVD risk (53).

Larger HDL particles, particularly the cholesterol-rich HDL-2 subfraction, are believed to enhance reverse cholesterol transport more effectively than smaller, less buoyant HDL-3 (59) particles, thus conferring greater cardioprotective benefits, as indicated by several studies (61, 62). It has been observed that DHA supplementation leads to an increase in the HDL-2 subfraction, whereas HDL-2 subfraction is not affected by EPA supplementation (47, 62). This finding was confirmed by a recent systematic review (63).

3.2.3 Clinical Evidence on the Effects of n-6 PUFAs on CVD

Evidence on the cardiovascular effects of n-6 PUFAs, LA, the major dietary n-6 fatty acid, and its major metabolite, AA, remain inconclusive. Research has shown that moderate intake of LA, when substituted for saturated fatty acids, leads to reductions in total blood cholesterol and LDL-cholesterol levels (64, 65). This effect appears to stem from the upregulation of the hepatic LDL receptor (LDLR) gene and subsequent increase in LDLR protein expression, thus facilitating the clearance of circulating LDL from the liver (64). Furthermore, human dietary trials have not yielded evidence indicating adverse effects of LA intake on platelet aggregation, endothelial function (66), or surrogate markers for inflammation, such as high-sensitivity C-reactive protein (CRP) (67).

n-6 PUFA intake has been associated with lower risk of CVD in cohort studies. A meta-analysis examining n-6 PUFAs based on their levels in blood and adipose tissue revealed an association with a reduced risk of CVD (68). In 30 cohort studies

with follow-up periods ranging from 2.5 to 31.9 years, a total of 15,198 incident cardiovascular events were recorded among 68,659 participants. Higher levels of LA were significantly correlated with lower risk of total CVD, cardiovascular mortality, and ischemic stroke, with hazard ratios per interquintile range of 0.93 (95% CI, 0.88-0.99), 0.78 (0.70-0.85), and 0.88 (0.79-0.98), respectively. AA levels were not correlated with higher risk of cardiovascular outcomes (68). However, a limitation of the included studies was the fact that residual confounding might occurred. This is because LA intakes are higher on plant-based diets (Fig. 3.2).

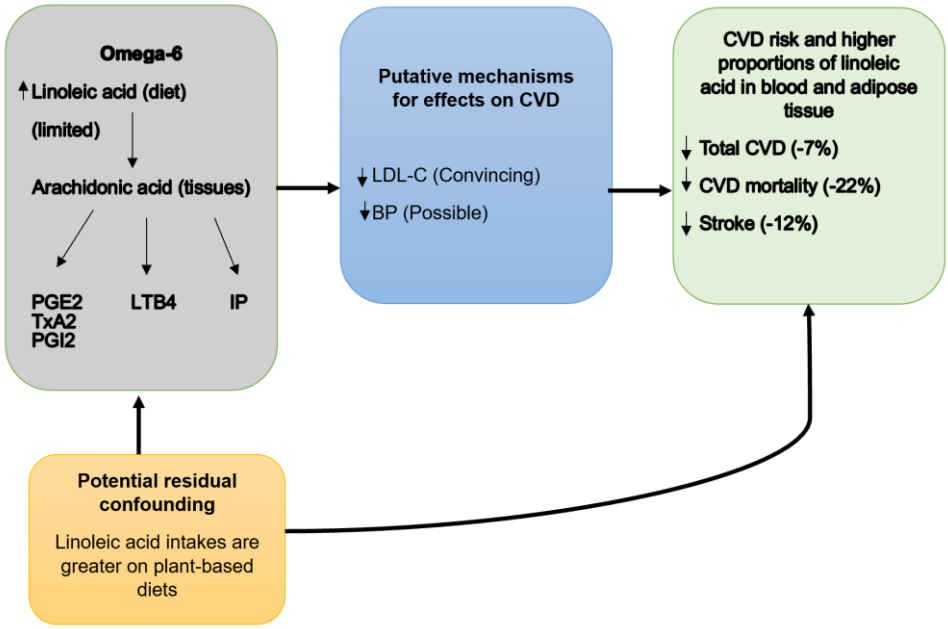

Fig. 3.2 Correlation between increased intake of linoleic acid with arachidonic acid and its metabolites; potential protective biological effects, and the correlation between higher proportion of linoleic acid in blood and adipose tissue levels with risk of CVD according to the meta-analysis by Marklund et al. (68).

3.3 Molecular mechanisms underlying the effects of n-3 and n-6 PUFAs

3.3.2 Incorporation of PUFAs into the lipid pool

n-6 and n-3 PUFAs are incorporated into phospholipids and TGs and are, therefore, key components of lipoproteins and building blocks of cell membranes and organelles. Consequently, the PUFA makeup of cell membranes and lipoproteins depends primarily on the fatty acid content of an individual's nutritional habits and everyday diet (29).

EPA and DHA exhibit differences in their metabolism and are found in different

quantities in different tissues, possibly reflecting differences in their function. The plasma levels of EPA and DHA increase in a dose-dependent manner following supplementation, reaching equilibrium within approximately one month (69). n-3 PUFAs are also incorporated in the sn-2 position of cell membrane phospholipids, where their maximum quantities after supplementation are reported within four to six months (69, 70). Supplementation with DHA leads to elevated EPA plasma concentrations, possibly resulting from slow EPA turnover, or retro-conversion of DHA. On the other hand, the rate of conversion of EPA to DHA is very low (69, 71) (Fig. 3.1). EPA is preferentially metabolized by lecithin-cholesterol acyltransferase, accumulating in cholesteryl esters in very low-density lipoproteins (VLDL) (72, 73). DHA, on the other hand, is predominantly incorporated into TG, possibly as a result of being a preferential substrate for diacylglycerol acyltransferase (DGAT) (73-76). The bioavailability and lipid incorporation of n-3 PUFAs also depends on the composition and formulation of the supplements used. Long-term supplementation with moderate doses of n-3 PUFAs (1.01g EPA/0.67g DHA) using re-esterified TG formulation was reported to lead to a higher red blood cell (RBC) membrane incorporation and a greater reduction in fasting plasma TG levels, compared to the ethyl ester formulation, in patients with dyslipidaemia on statins (77, 78). However, other studies investigating the effect of short-term supplementation with n-3 PUFAs as ethyl ester, free fatty acid, or re-esterified TG formulations, at low or high (>3g) doses, on incorporation into plasma lipids showed contradictory findings (79-82). The above evidence suggests that the lipid incorporation, and therefore the effectiveness of n-3 PUFA supplementation, is likely dose- and time-dependent, but also dependent on the composition and formulation of the supplement regimen used.

LA is present in high concentrations in the plasma, where it is incorporated into LDL and HDL, whilst it shows lower abundance in RBCs and platelets. In lipoproteins, LA is predominantly found in cholesteryl esters, but is also found incorporated in phospholipids and TGs. On the other hand, AA is primarily found in RBCs and platelets (83). EPA and DHA taken from the diet or supplementation can replace AA within cell membranes, particularly in leukocytes, RBCs, platelets and hepatocytes (84). Conversely, the incorporation of fish oil-derived EPA into neutrophil cell membranes was shown to be inhibited in healthy subjects on a LA-rich diet (85). This evidence suggests that competition between n-6 and n-3 PUFAs may determine their relative abundance in cell membrane and lipoproteins (Fig. 3.1).

3.3.3 Effects of PUFAs on lipoprotein metabolism

n-3 PUFA supplementation in the form of fish oil or a combination of pure EPA and DHA, has been shown to attenuate plasma TG levels and improve dyslipidemia (56, 78, 86). These effects are mediated by a number of molecular mechanisms. Specifically, EPA and DHA act on hepatocytes to suppress TG synthesis and VLDL production, as well as by promoting degradation of apolipoprotein B (87-90).

Inhibition of lipolysis in adipocytes by n-3 PUFAs also restricts the availability of non-esterified fatty acids for synthesis of VLDL (91, 92). Furthermore, induction of lipolysis by DHA and EPA leads to accelerated chylomicron clearance (93). Although an increase in HDL levels in response to n-3 PUFAs supplementation has been reported, this has not been confirmed by other studies (94).

Studies investigating the effect of supplementation with high doses (>3g/day) of different formulations of DHA or EPA for four to seven weeks reported varied results, with some showing a greater efficacy of DHA, whilst others no difference between the two n-3 PUFAs on the plasma lipid profile of healthy or hypertensive subjects (93, 95-98). Meta-analysis of the above studies indicated that DHA has an overall greater efficacy in reducing TG and increasing LDL and HDL cholesterol levels, compared to EPA (99). ComparED, a randomised cross-over study comparing the effect of 2.7g/day supplementation with DHA and EPA, in re-esterified triacylglycerol form, in subjects with abdominal obesity and subclinical inflammation, reported a greater efficacy of DHA in lowering TG and increasing HDL- and LDL-cholesterol levels. Furthermore, DHA increased apolipoprotein B levels, an effect not observed with EPA (100). As high doses (3-4g/day) of EPA or DHA were reported to increase liposome lipase activity with a similar efficacy, the increased efficacy of DHA is possibly not due to a more efficient clearance of VLDL and chylomicron (93, 101). Interestingly, the increased efficacy of DHA in reducing TG, observed in the ComparED study, was attributed to a greater proportion of study subjects responding to DHA compared to EPA (102).

n-3 PUFAs have been shown to inhibit DGAT, the enzyme catalyzing the terminal step of TG synthesis (103). n-3 PUFAs inhibit de novo fatty acid and TG synthesis in the liver, and promote fatty acid oxidation and TG catabolism in the muscle and adipose tissue by regulating the activities of transcription factors, sterol regulatory element-binding protein (SREBP)-1 and peroxisome proliferator-activated receptors (PPARs) (104, 105). SREBPs are activated by fatty acids, through proteolytic cleavage from the endoplasmic reticulum (ER) and translocate to the nucleus where they activate lipogenic gene transcription. SREBP-1 gene encodes two isoforms, SREB-1a and SREB-1c, through transcriptional regulation by different promoters, and alternative splicing (106). n-3 PUFAs prevent the proteolytic release of SREBP-1 from the ER, inhibiting its activation. Moreover, n-3 PUFAs promote the degradation of SREBP-1 mRNA and compete with the liver X receptor/retinoid X receptor (LXR/RXR) heterodimer, a transcriptional activator of SREBP-1 gene, inhibiting its gene expression (107-109). PPARs, are nuclear receptors that form heterodimers with RXR and bind to intracellular fatty acids species, acting as lipid sensors. The isoforms PPARα, primarily found in the liver, and PPARβ and δ, predominantly expressed in the skeletal and cardiac muscle, promote the transcription of genes involved in mitochondrial and peroxisomal fatty acid oxidation. PPARγ, on the other hand, regulates adipocyte differentiation, fatty acid oxidation and lipoprotein lipase activity. n-3 PUFAs promote fatty acid oxidation and lipoprotein lipase gene activation by acting as ligands for all PPAR isoforms

(110-112).

EPA inhibits DGAT activity more strongly than DHA in rat hepatocytes (113). Moreover, an *in vitro* study reported that EPA inhibits the binding of LXR/RXR to the SREBP-1c gene promoter more strongly than DHA in human embryonic kidney cells (107). *In vitro* studies in rat hepatocytes show that although both EPA and DHA induce PPARα activation and fatty acid oxidation, DHA promotes peroxisomal fatty acid oxidation as it is oxidised in microsomes, whilst EPA drives mitochondrial oxidation because it is oxidised both in microsomes and mitochondria (74, 114). However, a randomised control trial showed that EPA (3g/day) augmented lipogenesis without affecting plasma TG levels, whilst DHA (3g/day) did not affect the lipogenic index but significantly attenuated TG levels in young healthy subjects (101).

n-6 PUFA supplementation has been associated with reduced total cholesterol levels, possibly due to a reduction in LDL-C (67, 115, 116). A number of molecular mechanisms have been proposed for this effect. LA has been shown to increase the hepatic expression of the LDLR, leading to increased uptake of LDL and thus reduced plasma cholesterol levels (117). An n-6 PUFA-rich diet was also reported to increase LDLR levels by reducing the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein that promotes LDLR degradation, and to reduce the production of apolipoprotein B-100 (118, 119). As with n-3 PUFAs, n-6 PUFAs also promote lipolysis and fatty acid oxidation by activating PPARα-mediated signalling and inhibit lipogenesis by inhibiting LXR/RXR binding to the SREBP-1c gene promoter (107).

3.3.4 Effects of PUFAs on inflammation

n-3-PUFAs may also exert their anti-atherogenic effects by inhibiting vascular inflammation. DHA and EPA have been shown in randomised control trials to reduce inflammatory markers, such as IL-6, TNFα and CRP (120). The two n-3-PUFAs were shown by meta-analysis of randomized control studies not to have significantly different effects on blood inflammatory markers (121).

PUFAs are released from phospholipids by phospholipases and are metabolised into the lipid mediators oxylipins by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 (CYP450). Specifically, n-6-PUFAs, including AA, are metabolized into eicosanoids, such as the 2-series prostaglandins and thromboxanes, and the 4-series leukotrienes, which have inflammatory properties. On the other hand, EPA is converted to the less inflammatory 3-series prostaglandins and thromboxanes, and 5-series leukotrienes. EPA and DHA also give rise to anti-inflammatory mediators, such as resolvins, protectins and maresins (Fig. 3.3) (122-124). Competition between n-3 PUFAs and AA for metabolic enzymes, may thus lead to attenuated pro-inflammatory eicosanoid production. This is reflected in findings from clinical studies that show elevated plasma levels of n-3 PUFA-derived

anti-inflammatory oxylipins, and lower n-6-derived eicosanoids following moderate- or high-dose EPA and DHA supplementation (125-127). Gene polymorphisms leading to changes in CYP450 catalytic activity, and the specificity of CYP450 isoforms for specific n-3 PUFAs, may lead to tissue-dependent and inter-individual variability in the anti-inflammatory effects of EPA and DHA (128, 129).

n-3 PUFAs also suppress inflammation by acting on signalling pathways that regulate inflammatory/anti-inflammatory mediator balance. DHA and EPA inactivate the pro-inflammatory transcription factor nuclear factor (NF)-κB and induce the production of the anti-inflammatory hormone adiponectin in adipocytes, through PPARγ activation (130-132). The G-protein-coupled receptor free fatty acid receptor 4 (FFAR4/GPR120), which is primarily expressed in adipocytes and macrophages, also mediates the anti-inflammatory effects of n-3 PUFAs. FFAR4 activation leads to recruitment of the scaffold protein β-arrestin-2 and the formation of a FFAR4/β-arrestin-2 complex, which inhibits NF-κB activation and the formation of the NOD-like receptor protein 3 (NLRP3) inflammasome protein complex in macrophages. The NLRP3 inflammasome triggers the activation of the inflammatory cytokines IL-1β and IL-18 in response to metabolic stress, such as cholesterol crystals (133-135).

Evidence from *in vitro* studies demonstrates that EPA and DHA have differential effects on inflammatory signalling pathways. NF-κB activity was shown to be suppressed by EPA or DHA through two different signalling pathways in monocytes (136). EPA and DHA were also shown to bind to FFAR4 with different affinities leading to inhibition of NF-κB with the same efficacy but with different kinetics in human colon carcinoma cell line (137). Furthermore, EPA activates both calcium and β-arrestin-2-induced mechanisms, whereas DHA shows only weak activation of β-arrestin-2 in colon carcinoma cell line HT29 (138). The FFAR4 gene shows alternative splicing leading to a short form that triggers both calcium and arrestin-mediated cascades, and a long form that only drives arrestin-mediated signalling (139). Therefore, the relative abundance of each isoform in different cell and tissue types, and their regulation in disease, may contribute to the differential effects of EPA and DHA on FFAR4-dependent signalling. Differential regulation of PPARγ was also demonstrated in a rat model of myocardial infarction, where EPA exhibited a stronger anti-inflammatory effect by promoting a higher PPARγ activity (140).

The pro-inflammatory nature of AA-derived eicosanoids would suggest that n-6 PUFA supplementation may increase inflammation. However, clinical studies have shown that dietary n-6 PUFA intake and plasma levels inversely correlate with blood inflammatory mediator levels (141-143). This may reflect the action of a number of anti-inflammatory AA-derived eicosanoids (144). A low conversion of dietary LA to AA, and differences in the activities of enzymes involved in long-chain PUFA and eicosanoid production may also play a role (145). Specifically, the effect of LA supplementation on the proportion of plasma and lipid AA and the eicosanoid profile in healthy men was shown to be affected by single nucleotide polymorphisms in the fatty acid desaturase 1 (FADS1) gene (146). Furthermore, clinical studies have

shown that AA supplementation does not increase circulating inflammatory marker levels in healthy individuals (147, 148).

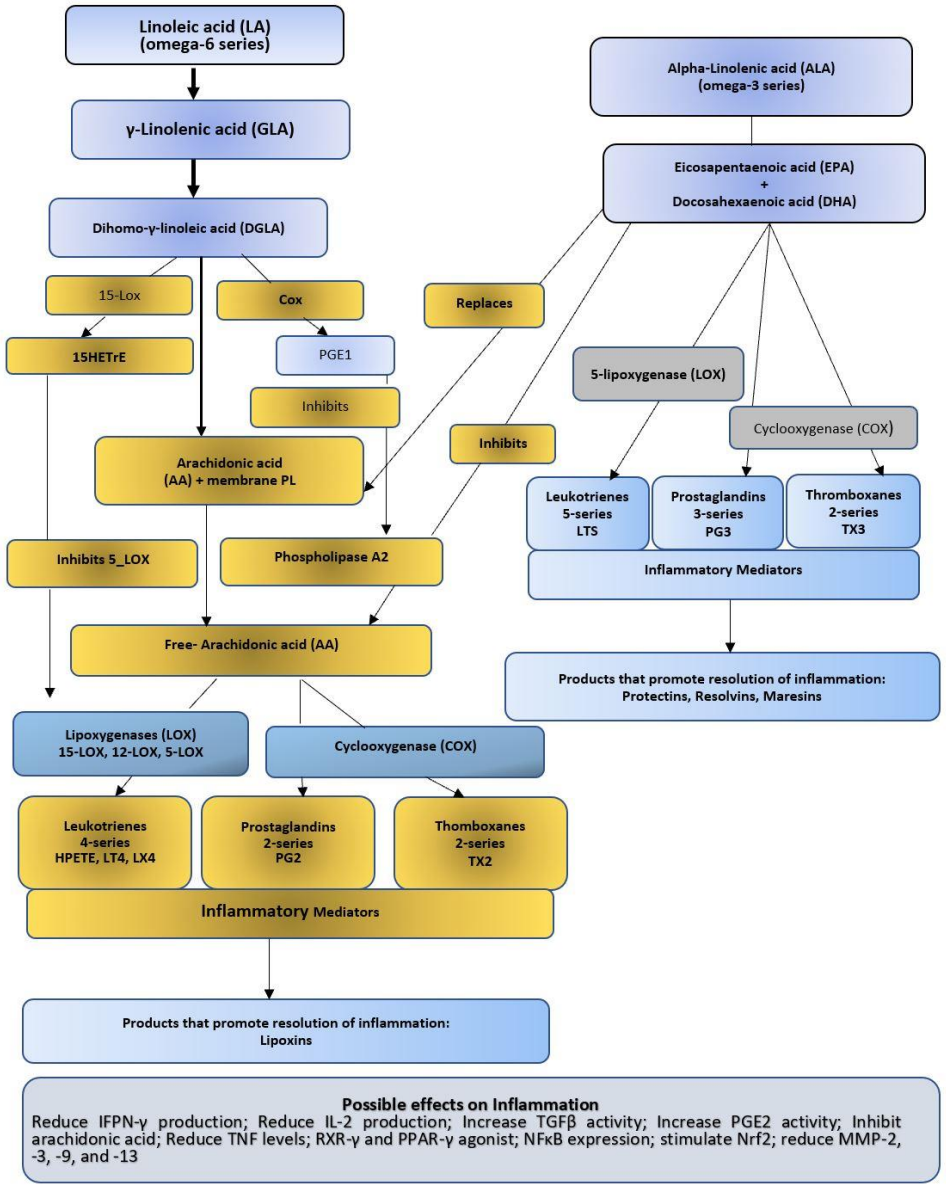

Fig. 3.3 Omega-6 and omega-3 PUFA consumption through diet; metabolic pathways of omega-6 and omega-3 PUFA series. Possible effects on inflammation. Adapted from Pantzaris et al. (149).

3.3.5 Effects of PUFAs on LDL oxidation

Dyslipidemia is associated with oxidative stress, which leads to LDL oxidation. Oxidized (ox)LDL promotes endothelial activation and recruitment of macrophages, which engulf LDL, including oxLDL, leading to the formation of foam cells. Foam cells promote vascular inflammation and destabilization of atherosclerotic plagues. Oxidative stress also leads to the formation of cholesterol crystal domains within endothelial cell membranes, which drive local immune responses through activation of the NLRP3 inflammasome (150, 151). EPA and DHA, and products of their oxidation, act as direct scavengers of reactive oxygen species but can also induce endogenous antioxidant mechanisms by activating nuclear factor erythroid 2–related factor 2 (Nrf2), a cytoprotective transcription factor (152-155). *In vitro* studies demonstrated a more potent and prolonged protective effect of EPA against oxidation of LDL and formation of cholesterol crystal domains, compared to DHA, which may reflect a more efficient electron stabilization due to its hydrocarbon chain length and double bond location (156, 157). On the other hand, clinical and animal model studies have demonstrated that LA-rich diets lead to enhanced susceptibility to LDL oxidation (158, 159). In line with this evidence, an *in vitro* study reported that, in contrast to EPA, LA and AA fail to inhibit LDL oxidation and cholesterol crystal domain formation (156).

3.3.6 n-3 PUFAs and epigenetic mechanisms

There is evidence suggesting that levels of n-3PUFAs, particularly of DHA, may influence gene expression through epigenetic changes, and specifically through modulation of DNA methylation. DNA methylation involves transfer of a methyl group from the methyl donor S-adenosyl methionine to a cytosine nucleotide, within a cytosine-guanine (CpG) sequence, by DNA methyltransferase enzymes, causing inhibition of gene transcription. S-adenosyl methionine also donates methyl groups to phosphatidylethanolamine-DHA leading to its conversion to phosphatidylcholine-DHA, which is the form in which DHA is delivered from the liver to the plasma and peripheral tissues. Therefore, changes in DHA levels may alter the availability of methyl groups for DNA methylation (160, 161).

Levels of dietary n-3 PUFA supplementation have been reported to be associated with altered DNA methylation patterns in clinical studies. A study in the Yupik native population in Alaska, who follow a traditional diet rich in fish and seafood, reported an association between n-3 PUFA intake and the differential methylation of 27 CpG sites in genes associated with inflammation and oxidative stress in the blood (162). In a randomized control study, supplementation of healthy subjects or patients with chronic kidney disease with 4g/day n-3 PUFAs (1.8g EPA, 0.2g DPA, 1.5g DHA ethyl esters) for eight weeks, was shown to be associated with changes in DNA

methylation in the promoters of the genes encoding for Δ6 desaturase and elongase 5 in peripheral blood mononuclear cells (PBMCs) (163). Similarly, PBMCs from obese subjects, on a low-calorie diet and n-3 PUFA-rich fish oil supplementation for eight weeks, showed altered CpG methylation in the gene encoding for CD36, a membrane glycoprotein involved in macrophage uptake of lipids, including oxLDL; however, the small magnitude of these changes indicates that they may not impact on gene expression (164). Similarly, 9-month-old infants who received cod-liver oil, rich in EPA and DHA, for 9 months did not show significant alteration in their blood leukocyte global DNA methylation levels, although CpG in specific genes were differentially-methylated (165).

There is also an increased interest in the effects of prenatal n-3 PUFA supplementation on epigenetic regulation of gene expression, and consequently on the development and health of the infant. Supplementation with DHA-rich fish oil (800mg/day) from week 20 of gestation until birth did not have a significant effect on the infant's blood leukocyte global DNA methylation levels, but promoted small changes in the DNA methylation of specific genes involved in neuronal development, appetite regulation, cell membrane lipid composition and immune responses (166). Furthermore, DNA methylation of the imprinted genes insulin-like growth factor 2 (IGF2) and H19 were found to be modulated in umbilical cord samples of infants, following maternal supplementation with DHA (400mg/day) from weeks 18-22 of gestation until birth. Specifically, DHA supplementation was associated with IGF2 hypermethylation and H19 hypomethylation (167). As IGF2 hypomethylation and H19 hypermethylation at birth have been associated with a higher risk of children being overweight or obese, prenatal n-3 PUFA supplementation may reduce the risk of metabolic and cardiovascular disease in children (168, 169).

The current evidence suggests that n-3 PUFAs influence the epigenetic landscape by regulating DNA methylation. However, the small magnitude of these changes suggest that they may not have a significant biological effect. On the other hand, this may be a result of sub-optimal n-3 PUFA doses, small study sample size and the fact that most study only study DNA methylation in the blood and not in other tissues. Therefore, more studies will be required to better understand the epigenetic effects of n-3 PUFA.

Conversely, genomic or epigenomic mechanisms may determine the response of individuals to n-3 PUFAs. This notion is supported by findings by the ComparED study showing that high doses of DHA and EPA exerted significant triglyceride-lowering effects only in a proportion (26%) of patients, despite compliance being close to 95% (102). This inter-individual variability has been attributed to polymorphisms in lipid metabolism and triglyceride synthesis genes, however, it is possible that epigenetic regulation may also play role (170-173).

3.4 Experimental evidence of the impact of the n-6/n-3 PUFA ratio on atherosclerosis

Given the clinical evidence on the role of n-6/n-3 PUFA ratio in the development of atherosclerosis and cardiovascular disease, a number of studies have investigated the impact of tissue n-6 PUFA background levels on the protective effects of n-3 PUFAs using *in vivo* disease models. Studies of the effect of modulating the dietary n-6/n-3 PUFA ratio on rats on a high-fat diet, have shown that a 1:1 ratio led to a greater reduction in serum TG, LDL, cholesterol, inflammatory cytokine and ox-LDL levels compared to higher ratios (174, 175). Commonly used models are the apolipoprotein E-deficient (ApoE-/-) or ApoE-/-/LDL receptor-deficient (ApoE-/-/LDLR-/-) mice, which spontaneously develop atherosclerosis (176, 177). ApoE-/-/LDLR-/- mice fed a diet containing a low n-6/n-3 PUFA ratio were shown to have lower plasma TG, LDL and total cholesterol and attenuated atherosclerosis progression, compared to diets with a higher n-6/n-3 ratio (178). Using ApoE-/- mice engineered to express the Caenorhabditis elegans gene fat-1 (ApoE-/-/fat-1), which encodes for a n-3 fatty acid desaturase that converts n-6 to n-3 PUFAs, were shown to have a lower n-6/n-3 PUFA ratio in plasma, monocytes and vascular tissue, compared to ApoE-/- mice on an identical n-6-rich diet (179, 180). Using this model, Wan et al have demonstrated that lowering the n-6/n-3 PUFA ratio leads to a reduction in NF-κB expression, production of inflammatory lipid mediators and cytokines, vascular recruitment of macrophages and development of atherosclerotic lesions (180). In an *in vitro* study using monocyte-derived macrophages, a reduction in AA/EPA ratio in the cell culture medium was shown to cause attenuated cholesterol uptake and increased cholesterol efflux, and to inhibit foam cell formation (181).

3.5 Conclusions

CVD stands as the predominant cause of mortality globally. Epidemiological investigations and randomized controlled trials have reported the potential of n-3 PUFAs to mitigate cardiovascular events. Notably, the evidence supporting the efficacy of n-3 PUFAs is more robust in secondary prevention contexts compared to primary prevention settings. Various mechanisms underpinning the cardioprotective properties of n-3 PUFAs have been proposed, with their impact on blood lipids emerging as the most scientifically-proven. Their most consistent effect is the reduction of serum TGs, but also minimizing the inflammatory effects. Nevertheless, conflicting findings regarding their clinical benefits have led to uncertainty regarding their utility in atherosclerosis prevention. Evidence on the cardiovascular effects of n-6 PUFAs remains inconclusive. However, n-6 PUFA intake has been associated with lower risk of CVD in cohort studies. Higher levels of LA were significantly correlated with lower risk of total CVD, cardiovascular mortality, and ischemic stroke. AA levels were not correlated with higher risk of cardiovascular outcomes.

Together with previous research, these results support the CVD benefits of LA and LC n-3 PUFAs (EPA and DHA). A balanced nutritional intake to maintain an optimal n-6/n-3 ratio is therefore of paramount importance in reducing CVD risk.

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Chapter 4

Untangling the Complexity of Cancer by Delineating the Signaling Mechanisms Forging Deadly Alliances within the Tumor Microenvironment

Maria A. Tsiarli and Constantinos Koumenis

Cite this chapter:

Tsiarli, M. A. and Koumenis, C. (2025). Untangling the Complexity of Cancer by Delineating the Signaling Mechanisms Forging Deadly Alliances within the Tumor Microenvironment. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 97-116). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 4
Untangling the Complexity of Cancer by Delineating the Signaling Mechanisms Forging Deadly Alliances within the Tumor Microenvironment

Maria A. Tsiarli[1] and Constantinos Koumenis[2]

1: Molecular Biology, Cell Biology and Biochemistry, Brown University, United States of America
2: Department of Radiation Oncology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America

Keywords: Cancer, tumor microenvironment, cancer neuroscience, glioblastoma, neonerves, cancer stem cells, integrated stress response, immune system.

Chapter Abstract: Technological progress has brought us unprecedented detail in the understanding of biological mechanisms underlying cancer development and progression, even beyond the level of single cell resolution. It is becoming increasingly clear that cancer evolves by co-opting processes with surrounding untransformed cells and biological systems which accelerate its evolution and promote survival, ultimately forming a “new” biological system with its own functional rules. In this chapter, we discuss the concept of the tumor microenvironment ecosystem with a particular focus on the contribution of the nervous system in brain and other solid cancers and the role of the Integrated Stress Response in promoting tumor cell adaptation to nutrient and hypoxia stress. We outline some of the current and emerging concepts and future directions that shape novel approaches in the development of therapeutics for cancer.

4.1 Cancer

Cancer is the second leading cause of death throughout the world, following cardiovascular pathologies. In 2020, there were 18.1 million new cancer cases, of which, a staggering 10 million resulted in death (Abdelaleem et al., 2019). Moreover, new cases of cancer are projected to reach over 35 million by 2050. These numbers amount to a 77% increase from 2022 (approximately 20 million new cases) and reflect a rapidly growing global cancer burden. Despite the fact that the primary underlying cause is the aging in the population, many new cases will include both childhood and young adult cancers and thus, urgently underscoring the necessity of taking groundbreaking steps to mitigate the toll of cancer.

Some of the key hallmarks of cancer include uncontrollable growth and reproduction of cells in a specific organ, which then acquire the ability to translocate and invade other organs, i.e., metastasize. The term cancer was first reported by ***Hippocrates***, who is considered the father of medicine, when he was describing breast cancer tumors, as resembling a crab whose feet radiate from its body. Thus, the term *karkinos* was born, from the Greek word for crab. Hippocrates claimed that the source of disease was an imbalance in the four bodily fluids (blood, black and yellow bile and phlegm According to the *humor theory*, *karkinos* formed in organs where accumulation of black bile took place. Maintaining the Hippocratic line of thought about cancer and its causes, the Roman ***Celsus***, translated *karkinos to the* Latin word for crab, *cancer*. Later, ***Galenos,*** used the word *oncos* (swelling in Greek) to describe tumors and *carcinos* for malignant tumors, from which the field of *oncology* was named.

With technological progress and ever-increasing understanding of how our body functions, came a better appreciation of the process leading to cancer development as well as the mechanisms it employs to evolve and spread from the original location to other organs. The nature and underlying mechanisms behind metastasis were the subject of many theories throughout the ages, with the prevailing line of thought being that cancer cells spread in the body randomly through the blood. Today, we know that metastasis is not a random event, but rather a process consisting of a series of well-orchestrated steps during which malignant cells migrate away from the main tumor mass, enter the vascular or lymphatic systems, survive in circulation, extravasate in distant tissues and organs and establish micro- and macro-metastatic nodules. It is also appreciated that interactions between the tumor cells and their surrounding biological systems, facilitate their spread and colonization of other areas of the body.

The first suggestion that the sites of cancer metastases are not completely random, was put forward by Dr. Stephen Paget, an English surgeon in 1989 (Fig. 4.1). Following post-mortem examinations of 735 women with breast cancer, he noted that the cancer always had a distinct metastasis pattern and was not random. This observation led him to postulate:

"*When a plant goes to seed, its seeds are carried in all directions; but they can*

only live and grow if they fall on congenial soil…While many researchers have been studying "the seeds", the properties of "the soils" may reveal valuable insights into the metastatic peculiarities of cancer cases".

And thus, the "*Seed and Soil*" Cancer theory was born. Paget's hypothesis was not taken seriously and was even challenged in 1928 by James Ewing, an American pathologist, who proposed that metastases are driven merely by mechanical variables of the circulatory system, *whereby* tumor cells are transported from one organ to another. It took more than half a century for the prevailing view of random metastases and tumorigenesis to change, when in 1984, trailblazing work by Isaiah Joshua Fidler, proved that metastases are not random. Specifically, *via* groundbreaking experiments, Fidler revealed the heterogeneity of tumor cell populations and importantly illustrated the unique relationship between tumor cells and organ microenvironments, which may define metastatic sites and by doing so, established the foundations of the tumor microenvironment field.

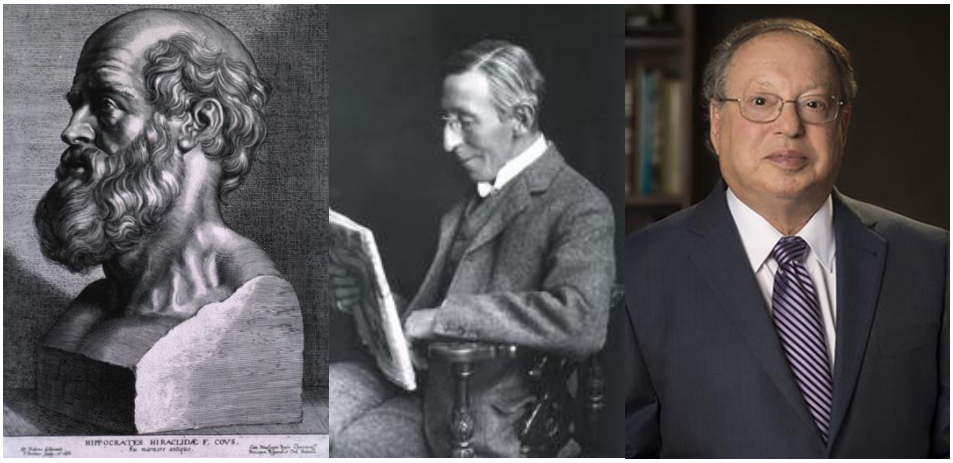

Fig. 4.1 The Seminal Figures in the Cancer Field: Hippocrates- Stephen Paget - Isaiah Joshua ('Josh') Fidler 1936–2020. Sources: Wikipedia- Courtesy of the National Library of Medicine: Wellcome Collection; Kerbel (2020)

4.2 The Tumor Microenvironment: A well-tuned biological system with profound influences on tumor behavior

Following Fidler's seminal contributions which shed light on the complexity of cancer behavior, the field of the *tumor microenvironment* bloomed. The term tumor microenvironment (TME) encompasses all the biological constituents and systems that surround, contribute to, and are interacting with a tumor. The TME includes multiple and heterogeneous cell populations and effector molecules, which differ depending on the tumor type and the tissue in context. Generally, the major TME components are cellular populations of infiltrating and resident host cells and the

non-cellular component of the extracellular matrix (Anderson & Simon, 2020). The TME represents a living ecosystem within which malignant and non-malignant cells establish complex signaling interactions that dictate tumor progression, metastasis, response to treatment and recurrence. The hallmark constituents of the TME include immune cells (e.g., T cells, macrophages, Tregs etc.), stromal cells (e.g. Fibroblasts, myofibroblasts, etc.), and endothelial and pericyte cells which make up the blood vessels and lymphatics (Hanahan & Weinberg, 2011). Along with secreted growth factors and cytokines, this collection of cells and the ECM usually exist in oxygen and nutrient-poor environments due to the abnormal and disorganized process of angiogenesis. These nutrient and hypoxic stresses created by the growing tumor, set up immunosuppressive and metabolically altered states that drive uncontrollable cancer cell proliferation and progression, aggressive behavior, metastasis, and treatment resistance (de Visser & Joyce, 2023).

A core characteristic of the TME is the multiple communication mechanisms between the intrinsic, extrinsic, and systemic components on the molecular and cellular level: cell-cell contact (PD-L1/PD-1); paracrine signalling (e.g. cytokines, chemokines, growth factors, proteases, extrallular vesicles, metabolites). This elaborate and multicomponent signaling scheme, results in the establishment of the TME as a selective pressure which culminates in promoting tumor growth and treatment resistance.

4.2.1 Stroma

The term *stroma* originates from the ancient greek word *στρῶμα*, meaning layer, bed or bed covering, appropriately as the anatomical stroma, is the part of the tissue or organ, providing a supportive role. Stromal support cells are heterogeneous, and the exact populations are tissue specific. Stromal cells mainly include cancer-associated fibroblasts (CAFs), tumour-associated vascular endothelial cells (TECs), pericytes (PCs), immune cells such as neutrophils, dendritic cells (DCs), lymphocytes, and monocytes, cancer-associated adipocytes (CAAs), mesenchymal stem cells (MSCs) and platelets (Anderson & Simon, 2020; Zhao et al., 2023). The stroma also consists of extracellular matrix (ECM), a network of secreted factors, such as collagens, glycoproteins, and other structural molecules. The structure and consistency of the ECM are tissue-specific and are critical factors in cancer metastasis, since TME-ECM interactions dictate the tissue biomechanical properties and allow for the enhancement of tumorigenesis and metastasis of cancer cells (Kai, Drain, & Weaver, 2019; Mouw, Ou, & Weaver, 2014). The tumor-surrounding ECM is mainly secreted and reconstructed by CAFs (Erdogan & Webb, 2017; Rodrigues, Heinrich, Teixeira, & Prakash, 2021).

Fibroblasts have been named the “healer cells”, as they are central in the wound healing response. Having this property, CAFs are key in successful tumorigenesis,

as they orchestrate remodeling of the extracellular matrix to accommodate for the growing tumor needs in space and nutrients, including allowing for the addition of tumor-associated vasculature. Furthermore, CAFs are major players in the modulation of the TME immune components such that they promote immunosuppression *via* the release of cytokines and chemokines (Mao et al., 2021). A critical aspect of tumorigenesis and acquisition of an aggressive and malignant phenotype by cancer cells is the process of epithelial to mesenchymal transition (EMT). EMT is a normally occurring phenomenon in development, wound healing, and tissue regeneration (Greenburg & Hay, 1982). Nonetheless, in the context of cancer is synonymous to aggressiveness, poor patient prognosis and poor response and/or evasion of treatment. Secretion of EMT promoting and pro-metastatic factors by CAFs causes tumor epithelial cells to relinquish expression of epithelial markers such as E-cadherin and adopt a mesenchymal phenotype by increasing expression of vimentin, N-cadherin, and fibronectin. These molecular changes are accompanied by loss of polarity, increased motility, initiation of metastasis and invasion to other organ sites *via* intravasation which is facilitated by macrophage-derived Vascular endothelial growth factor A (VEGFA) (Harney et al., 2015; Kai et al., 2019).

To obtain a deeper understanding of the interplay between intrinsic and extrinsic TME components and cancer cells, one needs to examine how the tumor stroma influences tumor cell aggressiveness and treatment resistance. Use of *in vivo* or *ex vivo* models such as organoids, is limited by the loss of the patients' stromal representation following engraftment. This in turn, results in loss of the spatial interrelationships between the stroma and TME components, and thus, does not allow for a realistic and complete characterization of the cancer cells' behavior. To overcome this challenge, patient-specific tumour TMEs are generated *in vitro* by reconstruction of individual components isolated from the patient's tumour (Mun, Lee, & Kim, 2024).

As a tumor outgrows the existing vasculature, decreasing gradients of oxygen develop due to diffusion limitations and continuous consumption of the available oxygen by the tumor cells. This results in the development of hypoxia, and acidosis in the tumors. The latter is due to the "Pasteur effect", that is the switch of the tumor cell metabolism from a primarily aerobic mode (relying on the TCA cycle for generation of ATP and energy) to an anaerobic one (generation of ATP primarily from glucose), which results in increase in lactate production (Dachs, Dougherty, Stratford, & Chaplin, 1997). During this process, cancer cells activate hypoxia-inducible transcription factors (HIFs) to compensate for the low oxygen levels and ensure tumor survival *via* altered gene expression and metabolic tuning (Talks et al., 2000; Wenger, Rolfs, Marti, Guénet, & Gassmann, 1996; Wouters, van den Beucken, Magagnin, Lambin, & Koumenis, 2004). Moreover, to compensate for the hypoxic levels the TME promotes mobilization of endothelial cells that initiate a process of vessel sprouting within the tumor, either by co-opting pre-existing vasculature or by stimulation of angiogenesis by inducing proliferation and increased survival of endothelial cells. This mechanism involves HIF-induced stimulation of

TECs, to secrete pro-angiogenic factors such as VEGF, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Moreover, endothelial cells are critical to the metastatic properties of tumors. Specifically, in a process orchestrated by tumor growth factor (TGF) and bone morphogenetic protein (BMP), endothelial cells undergo transformation, loss of basement membrane connection and become motile cancer-associated fibroblasts, a phenomenon known as "*endothelial–mesenchymal transition* (EndoMT)" (Zeisberg, Potenta, Xie, Zeisberg, & Kalluri, 2007).

4.2.2 Immune System

T cells are the most important executors of the immune response against the tumor, with $CD8^+$ T lymphocytes being at the forefront of the anti-tumor response. $CD8^+$ T cells *via* antigen recognition, identify tumor cells and kill them through secretion of cytotoxic molecules. On the other hand, regulatory $CD4^+$ T lymphocytes (Treg), act as pro-tumor agents by promoting immunosuppression through secretion of IL-10 and modulation of the antigen presenting cells, that are in charge for training naive T-cells into CD8+ anti-tumor cells (Giraldo et al., 2019). This in combination with other mechanisms employed by the tumor, such as reduced expression of major histocompatibility complexes (MHCs) and high immunosuppressing signaling and metabolic pro-tumor rewiring, results in promotion of immune evasion and the creation of an immunosuppressive TME (Kumagai, Itahashi, & Nishikawa, 2024).

Despite the impact of the c*ancer immunosurveilance theory* on cancer therapeutic discovery, which postulates that the adaptive immune system can constrain and regulate tumorigenesis, the relationship between the adaptive immune system and tumor incidence is far from a straight line. For example, we are learning that developmental mechanisms, that are hijacked by cancer cells, also work through dynamic and unexpected interactions with the immune system. Inflammation, which is directly linked to the function and activity of the immune system, has a direct role in developmental-stress-induced fate conversions. Specifically, single-cell omics analysis and functional assays in mouse embryos, revealed that trans-differentiation routes of EMT, EndoMT and endothelial-to-hematopoietic transition (EHT), are stimulated and directed by pro-inflammatory signaling mainly orchestrated by interleukins (IL-33) and Sp1 (Y. Zhang, Kang, Liu, Wang, & Liu, 2024).

The immune system is the gatekeeper of the body from internal and external threats, including cancer. Nonetheless, the role of the immune system in carcinogenesis is context/tissue- and cancer stage-dependent, as it functions as a double-edged sword by being either anti- or pro-tumorigenic, largely dependent on the stage of the malignant progression. Boasting a wide array of cells with different specializations, the immune system has proven to be both key to cancer therapy and a hindrance, as in some situations it promotes cancer progression, metastasis, and

resistance to therapy. Major immune cell constituents of the TME are T cells, B cells, natural killer (NK) cells which are the major effectors of the adaptive immune response; and macrophages and neutrophils which mediate the innate immune response.

The understanding of the fine communication and signaling details on the immune system and TME, holds great promise in the fight against cancer. This has been evident by the revolution brought upon cancer treatment by the introduction of cancer immunotherapies and specifically, of Immune Checkpoint Inhibitors (ICIs) that target T cell activity. Specifically, specific cell-surface receptors, such as CTLA-4 and PD-1 are specialized proteins that control T cell activity following an immune response (Ledford, Else, & Warren, 2018) to protect healthy cells from being attacked by an overactive or prolonged immune system activation. These proteins have become the focus of a number of therapeutic approaches, with several antibody or small molecule inhibitors being developed to block, or remove these "immune breaks" in malignancy, where a robust anti-tumor response is desirable. The recognition of the importance of immunotherapies in the fight against cancer was highlighted with the 2018 Nobel Prize in Medicine to James Allison at the University of Texas MD Anderson Cancer Center in Houston and Tasuku Honjo at Kyoto University in Japan, as to protect healthy cells from being attacked by the immune system. The recognition of the importance of immunotherapies in the fight against cancer was highlighted with the 2018 Nobel Prize in Medicine to James Allison at the University of Texas MD Anderson Cancer Center in Houston and Tasuku Honjo at Kyoto University in Japan (Ledford et al., 2018). Allison and Honjo exemplified how the immune system can be directed to attack cancer cells, by modification of immune cell proteins, thus, paving the way for immunotherapies that have undoubtedly revolutionized cancer therapeutics. Allison's work identified the CTLA4 ICI protein, which led to the creation of the first ICI antibody authorization in 2011, namely *Ipilimumab*. In recent years came the approval of *Pembrolizumab* and *Nivolumab* immune checkpoint inhibitors blocking PD-1 (T-cell surface protein), and of *Atezolizumab* and *Durvalumab* anti-PDL-1 (cancer cell surface protein) antibodies. ICIs have been used alone or in combination with chemotherapies for the treatment of over 50 cancer types including colorectal cancer, melanoma, breast cancer, non-small-cell lung carcinoma, bladder cancer and renal cell carcinoma, while ongoing more than 3000 clinical trials ongoing for other types of cancer (Robert, 2020; Yu, Hubbard-Lucey, & Tang, 2019)

In addition to ICIs, another widely employed therapy, especially for blood-related cancers is the Chimeric Antigen Receptor (CAR) T-cell therapy. In this approach, T cells are extracted from the patient and modified in the lab with the addition of surface proteins that act as sentinels of tumor-associated antigens on cancer cells which they bind, and by doing so, allows tumor cell destruction by the chimeric T cells. The modified T-cells are infused back into the patient. The first CAR-T treatments were created for the treatment of childhood blood cancers, namely acute lymphoblastic leukemia (Maude et al., 2018; Milone et al., 2021) . In very recent

developments, CAR-T therapy has been applied for glioblastoma, a solid tumor, with promising initial results of initial tumor shrinkage. Unfortunately, the tumors reappeared in different time points for each of the participants, suggesting more fine details of the mechanisms must be understood to achieve a long lasting and effective treatment (Bagley et al., 2024; Ledford, 2024). Immune checkpoint blockade treatments with monoclonal antibodies, specific for immune cell receptors, have been used for many types of solid and hematologic malignancies and have shown many promising and lifesaving results (Robert, 2020).

4.2.3 The integrated Stress Response

Cellular stress can be the result of different factors and causes dysregulation of cell homeostasis. To control stress, eukaryotic cells have developed the *integrated stress response* (ISR), of which the effector is the eukaryotic translation initiation factor 2 (eIF2). The ISR cascade is initiated by phosphorylation of the α2 subunit of eIF2 on serine 52 and followed by induction of gene expression, including of *ATF4*. The result of ISR activation is a pro-survival, homeostatic program, or apoptosis, if the stress dynamics overpower cell survival (Ljujic et al., 2016). The TME, is characterized by the presence of multiple forms of stress, including hypoxia, nutrient and amino acid deprivation, acidosis, and oncogenic activation of cancer cells. Thus, to overcome these stressors, the TME impinges on tailored ISR responses that promotes tumor survival (Roby et al., 2022). In KRAS-driven lung cancer phosphorylation of eIFa2 is associated with increased tumor cell proliferation, invasiveness and poor prognosis and outcome (Ghaddar et al., 2021). In experimental melanoma and pancreatic tumour models, ATF4 expression in CAFs has been shown to regulate TME angiogenesis and ECM consistency *via* the production of collagen (Verginadis et al., 2022). This pro-tumor and angiogenic effect of CAFs is eliminated when ATF4 is genetically ablated. ATF4 has also been implicated in the neovascularization mechanism seen in glioblastoma (GBM) brain tumors. Specifically, ATF4 regulates phosphoglycerate dehydrogenase expression, a key factor in endothelial cell metabolism and results in aberrant vascularization patterns for which GBM is notoriously known for and hinder immunotherapies (D. Zhang et al., 2023).

4.3 The Nervous System: a new dimension to the TME

The nervous system (NS) is of vital importance for proper biological functioning. Nerve endings are spread throughout the body and organs, like a finely spun spider web. Exceptions to this are nails, hair shafts and the blood. Human bones show

variable patterns of innervation, with the periosteum being the most densely innervated unit, followed by the bone marrow and cortex. The thoracic vertebral bodies and parietal bones have the most and the least innervation, respectively (Steverink et al., 2021). It is intriguing to note the degree by which the NS orchestrates brain-body communication. A highlight of this exquisite level of organisation was the recent characterization of afferent innervation of adipose tissue (and not just efferent as it was previously thought), which suggests a complex communication system from the peripheral organs to the brain, contains spatial encoded information and entails ramifications that extend beyond sensory control of metabolic homeostasis up to interoception (Mishra & Townsend, 2023; Wang et al., 2022).

Despite the immense technological progress in science, we are still far from unlocking the secrets of the nervous system and the brain. As it is apparent, having a firm understanding of the NS efferent and afferent map, will allow us for more effective interventions in terms of disease and in general of wellbeing. Thus, it is not too surprising that in the past decade, the NS is becoming another critical piece of the TME puzzle, one which can orchestrate intricate communications between the stroma and the cancer cells (Monje et al., 2020; Winkler et al., 2023) (Fig. 4.2).

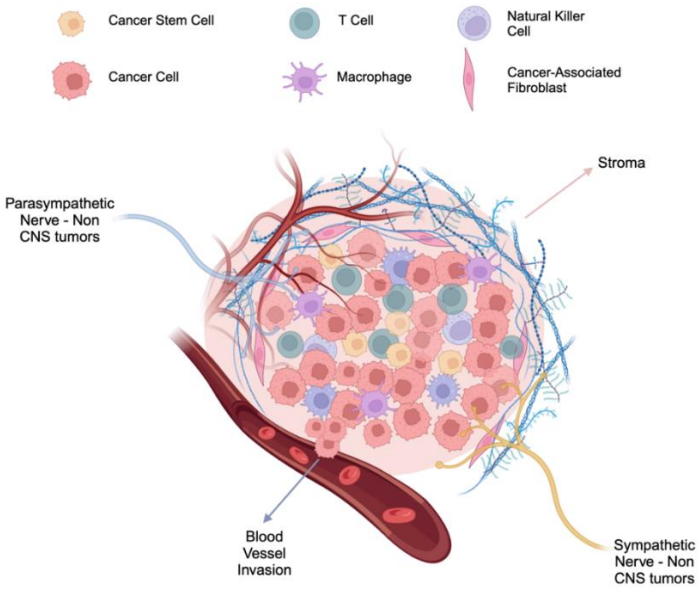

Fig. 4.2 The Tumor Micro-Environment (TME) (Created with BioRender.com)

4.3.1 How cancer cells hijack the NS

The NS regulates many biological functions and is a key contributor for the maintenance of cell homeostasis. The foundational discovery/realization that both

the central and the peripheral NS have a foundational role in cancer initiation and progression, brought a new paradigm shift in the cancer field and is revolutionizing the way we understand cancer mechanisms and the plasticity of the nervous system-cancer communication. This seminal discovery gave birth to the field of Cancer Neuroscience.

The interaction of the NS with disease, including cancer has been a subject of fascination since ancient times. According to the *Humor Theory* by Hippocrates, *melancholia* (from Greek word *μελαγχολία*: *μέλαινα* + *χολή,* melaena-chole, meaning black bile), was a result of excess black bile. Galen, a proponent of the Hippocratian thought, stated that *cancer* was a result of melancholia (Jackson, 1986). Following the seminal contributions of Paget and Fidler in elucidating that cancer evolution is intertwined with the environment in which it is growing and invading, i.e. the tumour microenvironment, more attention was given to contributions of other biological systems to carcinogenesis. Nonetheless, the NS was not, until only recently, considered to be a major player in this equation as more attention was given in the role of the immune system (Magnon & Hondermarck, 2023). In the following sections we will elaborate the discoveries that have gradually shed light on the importance of the NS in oncogenesis, which as the field progresses, appears to be the mastermind behind other biological systems-cancer interactions.

4.3.1.1 Perineural Invasion and Axonogenesis

In 1986 the Nobel Prize for Physiology and Medicine was awarded to Rita Levi-Montalcini and Stanley Cohen for the discovery of the nerve growth factor, a breakthrough that opened the window to how neuronal signalling can affect cancer evolution. Levi-Montalcini was collaborating with Elmer Bueker on neuroembryology experiments in which mouse sarcoma tissues were grafted into the body wall of chick embryos. These experiments revealed innervation of the neoplastic tissue by sensory nerve fibres from adjacent dorsal root ganglia. Bueker speculated that the neoplastic tissue possessed such histochemical properties that favoured and attracted the growth of sensory fibres (Bueker, 1948; Levi-Montalcini, 1987), reminiscent of the “seed and soil” hypothesis. Levi-Montalcini expanded these observations eventually leading her to the characterization of the nerve growth factor as the soluble factor that mediated the signalling attracting the nerve fibres and causing their hypertrophy. As a continuation of the above discoveries, came the contributions by Claire Magnon at the French National Institute of Health and Medical Research in Paris. Magnon characterized new nerve fibre growth, “*neonerves*”, sprouting throughout and around prostate tumors in mice, in a model where sympathetic neonerve sprouting promotes the early stages of tumorigenesis, and parasympathetic neonerves promote cancer dissemination (Magnon et al., 2013; Magnon & Hondermarck, 2023). This phenomenon was later documented in other

types of cancer such as skin, pancreas, and stomach (Magnon & Hondermarck, 2023).

Metastasis is the phenomenon by which cancer cells or more appropriately, malignant cancer cells, detach from the primary tumour site and translocate to other organ sites where they establish new tumorigenic colonies. Blood and lymphatic vessels were known to be used as metastasis channels and were densely studied, perhaps due to the previously held theories in cancer spread e.g. Humoral Theory and Lymph Theory of Cancer, the latter of which supported that fermentation of the lymph and its acidification was causing cancer (The American Cancer Society, 2014). Nonetheless, reports from the mid 18th century investigating head and neck cancers, stated tumour can also proceed through and along nerves (Cruveilheir, 1842; Liebig, Ayala, Wilks, Berger, & Albo, 2009; Neumann, 1862). Essentially, these early reports documented the phenomenon of *Perineural invasion (PNI)*, by which cancer cells surround and infiltrate nerve fibres (neoplastic invasion), by describing the tendency of these cancers to travel along nerves on their way to the intracranial fossa. We now know that PNI is associated with tumour aggressiveness, poor prognosis and cancer-associated pain (Chen et al., 2019). Seminal contributions to this phenomenon were made by Gustavo Ayala (Baylor College of Medicine in Houston, Texas), who documented nerve invasion in human prostate cancer cells and in biopsy samples (G. Ayala, 2023; G. E. Ayala et al., 2008). Moreover, Anil Sood (University of Texas MD Anderson Cancer Center in Houston), who was working on the relation of stress and cancer, documented that β-adrenergic receptors expressed by cancer cells bind the neurotransmitters epinephrine and norepinephrine which are secreted by peripheral nerves and are key effectors of the stress response. Through these interactions cancer growth and progression is stimulated by the NS in conditions of chronic stress (Thaker et al., 2006).

A hallmark of cancer is the extreme metabolic adaptation of cancer cells, by compensating nutrient poor conditions through use of alternative metabolic resources such as inducing nutrient release from TME components, including TAFs and macrophages. Another mechanism by which cancer employs to metabolically adapt in nutrient-deprived environments, is the release of nutrients by surrounding peripheral axons. Specifically in human cell lines of pancreatic cancer (PDAC) and in mice PDAC models, it was shown that cancer cell survival was promoted by release of amino acids, including serine by peripheral axons, which in turn induced PDAC cell signaling promoting further tumor innervation (Banh et al., 2020). This study highlighted the complex interplay between the nervous system and cancer which evolves in multiple levels, ranging from metabolic adaptation of cancer cells to restricted nutrient conditions to control of gene expression and translation favoring cancer growth *via* neuronal metabolic activity.

4.3.1.2 Highjacking of Neurodevelopmental Mechanisms and Active NS remodeling and Recruitment

Through these discoveries it has become apparent that cancer cells can adapt mechanisms from the components of the TME to sustain tumor growth and promote invasiveness. In 2019, an exciting new mechanism was added into the cancer-TME interactions. Specifically, Magnons' group revealed that neural progenitors originating from the subventricular zone of the central nervous system, travel through the bloodstream to infiltrate prostate tumours and metastases in mouse models. Upon their arrival at the tumor site, these progenitors are engaged into generation of new nerve fibers/neurogenesis (Mauffrey et al., 2019). Furthermore, in recent years, studies from independent groups revealed that cancer cells activate neurodevelopmental mechanisms to promote their survival and tumor growth. Specifically, glioma cells develop specialized neurite-like membrane protrusions called tumor microtubes (TMs), which have multiple functions including, invasion, metastasis, and proliferation of glioma cells. Furthermore, through these ultra-long protrusions glioma cells interconnect establishing and electrically active multicellular cancer network, that extends beyond the initial tumor sites to other brain areas and is one of the causes for glioblastoma treatment evasion (Winkler et al., 2023). Moreover, glioma cancer cells establish *bona fide* unidirectional glutamatergic synapses with surrounding neurons which signal *via* excitatory postsynaptic currents (EPSCs) mediated predominately by calcium permeable AMPA receptors (AMPAR) in glioma cells and promote their proliferation and tumor growth and increase in invasiveness. Glioblastoma cells connected *via* TMs, develop autonomous pacemaker activity via rhythmic Ca2+ signaling, similar to early neurodevelopmental signaling taking place during brain development, and *via* this mechanism tumor growth is further enhanced. A surprising discovery on the degree that glioblastoma cells can integrate into the normal brain and their surrounding TME, is the fact that activation of tumour-infiltrated cortex in GBM patients causes activation of cortical regions beyond what is normally expected in the healthy brain. This study showed that GBM induces neural circuit remodelling in the human brain, promoting tumour progression and cognition impairment. Plus, the degree of GBM infiltration in the normal brain is negatively correlated to survival (Krishna et al., 2023).

4.4 Conclusions and Perspectives

As outlined in the last section, the NS is a major component of the TME and a critical factor in tumor establishment, progression, and treatment evasion. The discoveries made thus far about the NS-TME-tumor, delineate that we have yet much to understand about the nature and the mechanisms employed in these interactions, both in the central NS and the peripheral NS. In summary we now know that (a) the

nervous system can be induced by a tumor to undergo remodelling both in the local TME and in distant locations; (b) the autonomic NS provides the vehicle by which brain activity can influence peripheral/distal tumors; (c) the NS-TME can regulate tumor immune responses and tumor immunity (Amit et al., 2024) and (d) tumor cells can hijack neurodevelopmental mechanisms to promote tumor growth, enhance tumor immune protection and evasion of treatment. These findings have paved the way for more mechanistic questions to better understand how these phenomena are established and perpetuated during the course of tumorigenesis. Furthermore, they have uncovered an overlooked aspect of cancer therapies, and specifically their neurotoxic effects and how these effects on the NS reflect on the NS-tumor-TME relationship as a whole. On the other side of the coin, the interdependency of nerves and cancer cells, opens the way for the repurposing of drugs for cancer that are traditionally used for neurological conditions (Abdelaleem et al., 2019; Shi et al., 2022).

The harnessing of the immune system to fight malignancy is fast becoming another major pillar in cancer therapeutics a. Despite the documented significant benefit of immune therapies for cancer, there are still key drawbacks related to them, namely toxicity related to off-target and on-target off-tumour effects (Xue et al., 2024). These side effects arise due to lack of spatial and temporal control of the treatments, and because they are usually administered in high doses in order to reach the tumor site. The development of spatial omics technologies is slowly allowing for a more precise and detailed understanding of the spatial inter-relationships within the components of the TME and of the mechanisms that are employed for tumor immunity. Furthermore, the combination of spatial technologies with AI applications, promises a more fast and translational approach from the bench to the patients' bedside (Walsh & Quail, 2023). In the quest to minimize side-effects and increase the effectiveness of immune therapies, various approaches have been developed and/or are explored. Namely, combination cancer immunotherapy, allows for a more personalized approach such as coupling of chemotherapy with immunotherapy, radioimmunotherapy, combination of small molecule inhibitors with CAR-T cells or use of cancer vaccines to prime T-cells against the tumor. Other non-traditional combination approaches are sonodynamic immunotherapy and photodynamic immunotherapy (Zhu et al., 2021). Furthermore, use of biomaterials responsive to either exogenous or endogenous cues such as light or pH, respectively, holds promise in better spatial and temporal control of the administered immunotherapy (Xue et al., 2024).

One of the biggest challenges in understanding cancer mechanisms and how the TME is implicated in nurturing cancer growth and therapeutic evasion, is the inherent heterogeneity of the tissue in context and of the TME *per se*. The TME heterogeneity spans different levels of organization. The cellular heterogeneity and cell type specialization especially, pose a great hurdle in understanding the details of signaling mechanisms, the signaling sequences that unfold during tumorigenesis and establishment of the TME (Swanton et al., 2024). Furthermore, a clear understanding

of the neural-cancer interactions in the TME is critical to improving our understanding of cancer mechanisms and provide new precise and personalized cancer treatments with less or minimal side-effects.

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Chapter 5
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Andreas Ch. Hadjichambis and Demetra Paraskeva–Hadjichambi

Cite this chapter:

Hadjichambis, A. Ch. and Paraskeva–Hadjichambi, D. (2025). The Pedagogical Approach of Education for Environmental Citizenship in Biology Education: An Innovative Learning for Sustainability. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 117-139). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 5
The Pedagogical Approach of Education for Environmental Citizenship in Biology Education: An Innovative Learning for Sustainability

Andreas Ch. Hadjichambis[1] **and Demetra Paraskeva–Hadjichambi**[2]

1: Cyprus Biological Society (CBS) & Cyprus Centre for Environmental Research and Education (CYCERE), Lemesos, Cyprus & Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus.
2: *Cyprus Centre for Environmental Research and Education (CYCERE), Lemesos, Cyprus & Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus.*

Keywords: Biology education, Education for Environmental Citizenship, Environmental Education, Environmental Citizenship, pedagogical approach.

Chapter Abstract In recent years, the enormous developments of the biological research have been a turning point for biological education and has provided enormous insight into the contribution of the biological education in the generic scientific literary. In addition, contemporary biology education has seen remarkable development nowadays and has emerged as a separate field in biological research. The progress from the early days of single knowledge-oriented biological education led to contemporary biological education that aims integrated biological literacy which is so essential in modern complex societies. This chapter provides the insights of contemporary biological education in relation to sustainability and the socio-ecological issues which are fundamental components of biological literacy. The chapter defines the Education for Environmental Citizenship, presents the pedagogical landscape, the characteristics, the notions and the competences of Education for Environmental Citizenship. In addition, the details of the Education for Environmental Citizenship Pedagogical Approach for an innovative learning for achieving sustainability are given. Finally, the chapter describes a case study from Cyprus which facilitates important empirical data, using the ECQ, a duly validated Questionnaire, already used in many international studies.

5.1 Introduction

Contemporary biological education includes topics such as ecology, biodiversity conservation, systematic botany and taxonomy, ecosystems and their management, population biology, endangered species, biogeochemical cycles, material recycling and energy flow, photosynthesis and pollution. These biological topics are of critical importance in the observed environmental and climate crisis and this crisis could be overcome through transforming education which consider the sustainable development goals – SDGs (Hudson, 2001). The biology education of the 21st century is necessarily interdisciplinary, system-oriented, includes quantitative skills, initiative problem solving, and integrative approach (Labov, Reid, & Yamamoto, 2010). Biological phenomena connected to socio-scientific issues, such as climate change and biodiversity loss, need to have a comprehensive and interdisciplinary approach to be thoroughly taught and learned. In this basis, biology education needs to include teaching methods such as experiential, collaborative, process-based and problem-based experimental learning and computer-assisted methods (Jeronen et al., 2016). Also, several biological topics require approaches promoting experimental problem-solving and process-based skills (Keselman, 2003; Ehmer, 2008). In addition, field-based activities, e.g., fieldwork and field trips, provide students with authentic and interactive experiences and experiential learning opportunities, which increase students´ interest and enhance their learning (Simmons, 2008). Students´ engagement in field-based activities plays a crucial role in learning biological issues. Fieldwork provides students the chance to observe the environment and to use scientific inquiry to test ideas and concepts they have learned in the classroom (Jeronen et al., 2016). According to Hart and Nolan (1999), fieldwork and outdoor learning beyond the boundaries of the classroom, have a positive effect on students´ knowledge, attitude and behavior, essential factors also in promoting sustainability.

5.2 Contemporary Biology Education and Sustainability

The biological topics mentioned in the introduction are just some of the common areas between biological education and education for sustainability and environmental education. The contemporary biology education and the SDGs will remain on the global scientific agenda for at least the next two decades and needs the appropriate emphasis (Wibowo & Saidikin, 2019). In addition, through urgent environmental and ecological topics such as the above, modern biological education is internationally called to move from simple knowledge and skills to levels of more complex and more substantial education and includes components such as the cultivation of attitudes, behaviors and values.

Semilarski and Laius (2021) pointed out that socio-ecological issues is a fundamental component of the biological literacy (Fig. 5.1). Sustainability, which consists of responsible decision-making and responsible behavior towards environment, is

crucial. To enhance a sustainability worldview, students should have the ability to act in such a way that their actions have a meaningful impact (Nolet, 2017). The dimension of sustainability involves global competences. Global competence is defined as multidimensional capacity, when people can examine local, global and intercultural issues, understand and appreciate different perspectives and world views, interact successfully and respectfully with others, and take responsible action toward sustainability and well-being (Organization for Economic Co-operation and Development (OECD), 2018). Global competences can promote people knowledge and skills towards sustainability. Biological literacy should develop students' critical awareness of human relationships with nature (Oliveira et al., 2019). Narguizian (2019) addresses that all educators should help students to understand of Human-Earth relationship, enabling the citizenship to cope creatively in their future lives (Semilarski and Laius, 2021). In addition, aspects enabling students to make theoretically justified bioethical choices, and make theoretically justified choices about their health and environment should be included in the biologically literate students' model.

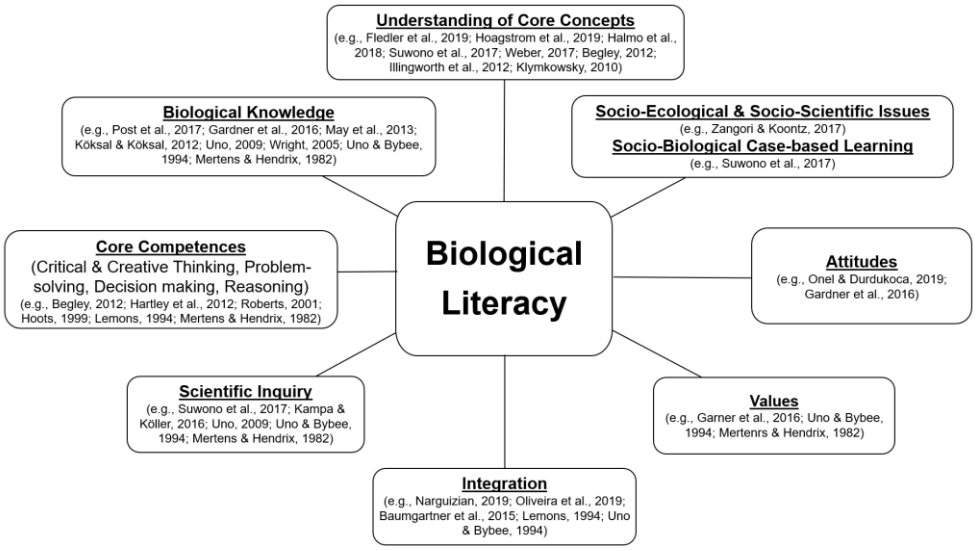

Fig. 5.1 Biological literacy modified from Semilarski and Laius (2021) content analysis.

The cultivation of environmental citizenship is a contemporary necessity in the observed environmental crisis, and modernized biological education can contribute a lot to this (Hadjichambis et al., 2020). Biological education needs today, more than ever before, to be able to empower environmental citizens who act and participate in society as agents of change in the private and public sphere, on a local, national, and global scale, through individual and collective actions, in the direction of solving contemporary environmental problems, preventing the creation of new environmental problems, achieving sustainability as well as developing a healthy relationship

with nature. Based on the above, the adoption of the pedagogical approach of education for environmental citizenship can be proved as an innovative learning to achieve sustainability.

5.3 The Education for Environmental Citizenship Pedagogical Approach

5.3.1 Defining Education for Environmental Citizenship

Education for Environmental Citizenship (EEC) is an innovative educational approach promoting pro-environmental behavior with individual and collective actions, in private and in public spheres and in local, national and global scales as well as engagement and civic participation. According to the European Network for Environmental Citizenship (ENEC), in which more than 160 experts from 39 countries participate (including European countries, USA, Australia and Israel), Education for Environmental Citizenship (EEC) can be defined as:

> "Education for Environmental Citizenship is defined as the type of education that cultivates a coherent and adequate body of knowledge as well as the necessary skills, values, attitudes and competences that an Environmental Citizen should be equipped with in order to be able to act and participate in society as an agent of change in the private and public sphere on a local, national and global scale, through individual and collective actions in the direction of solving contemporary environmental problems, preventing the creation of new environmental problems, achieving sustainability as well as developing a healthy relationship with nature. 'Education for Environmental Citizenship' is important to empower citizens to practise their environmental rights and duties, as well as to identify the underlying structural causes of environmental degradation and environmental problems, develop the willingness and the competences for critical and active engagement and civic participation to address those structural causes and act individually and collectively within democratic means, taking into account the inter- and intra-generational justice (ENEC 2018)."

The EEC definition can be integrated and illustrated in the EEC model (Fig. 5.2). In the core of the EEC Model is situated the green cycle which includes the necessary knowledge, values, attitudes, skills, competences, and behaviors that an environmental citizen should be equipped with in order to be able to act and behave as an agent of change. In addition, the other constitutional elements of the Education for Environmental Citizenship, which are Outputs (in orange arrows), actions' dimensions (individual and collective), spheres (private and public) and scales (local, national, and global), form the EEC Model. It should be clarified that the exact position of each output in the EEC Model does not illustrate its relationship with actions' two dimensions, two spheres and three scales.

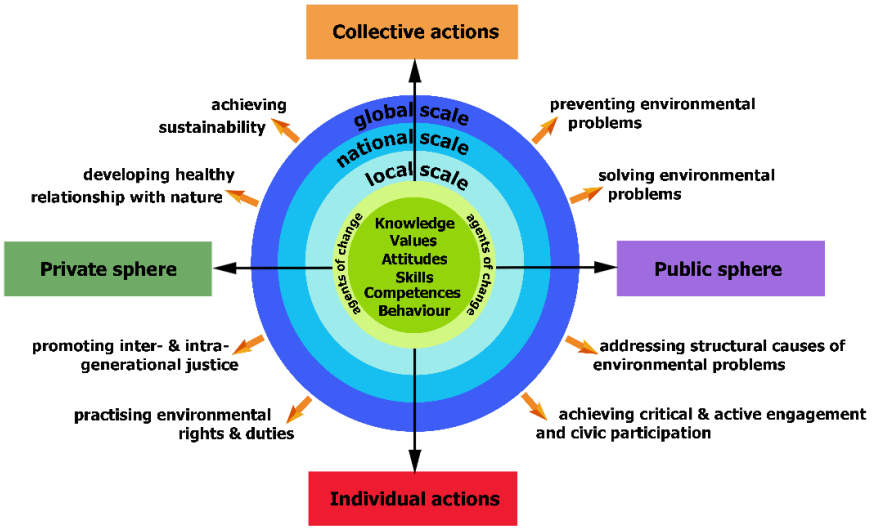

Fig. 5.2 Education for Environmental Citizenship Model (Source: Hadjichambis and Paraskeva-Hadjichambi, 2020)

The theoretical conceptualization of the EEC was deeply elaborated in previous scientific publications (e.g., Hadjichambis & Paraskeva-Hadjichambi, 2020) and interested readers could trace back to these works for a thorough theoretical background.

5.3.2 Pedagogical landscape of Education for Environmental Citizenship

EEC is the type of education that promotes Environmental Citizenship. It is considered essential to determine the pedagogical landscape in which EEC is placed. Some of the existing pedagogical theories and approaches are important for EEC because each of them contributes to some extent to its overall scope and goals. The following pedagogical theories and approaches (Fig. 5.3) are forming the pedagogical landscape of EEC: Place-based learning; Problem-based learning; Civic ecology education; Pedagogy for eco-justice; Action competence learning; Community service learning; Participatory action research; Socio-scientific Inquiry-based Learning. The referred pedagogical theories and approaches overlap considerably and their overlaps are possible components of the Education for Environmental Citizenship.

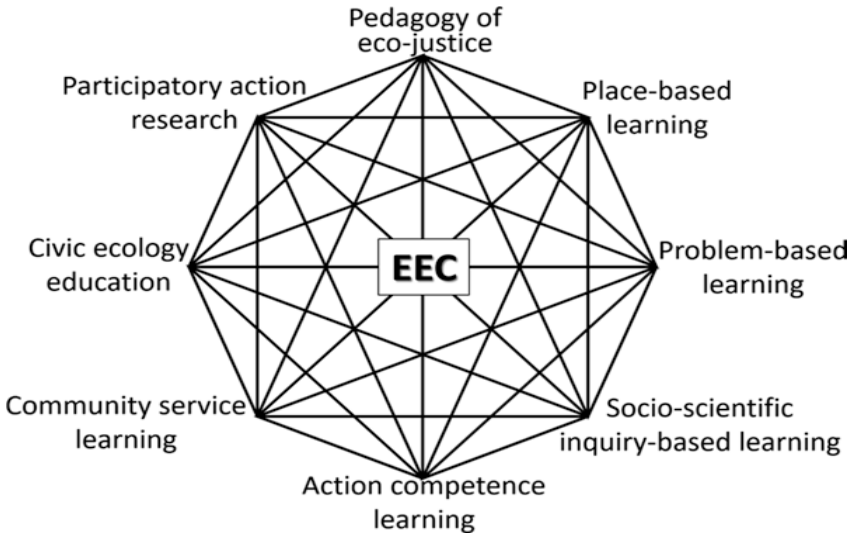

Fig. 5.3 The pedagogical landscape of Education for Environmental Citizenship (Source: Hadjichambis and Paraskeva-Hadjichambi, 2020)

The pedagogical theories and approaches mentioned above can make a significant contribution to EEC, however, none of them alone can promote the whole scope, the objectives of the EEC and its outputs.

5.3.3 Characteristics of Education for Environmental Citizenship

Education for Environmental Citizenship has some important characteristics which identifies its focus. It is obvious that EEC is a comprehensive and integrated learning which brings together important qualities of different individual theories and approaches. It combines what can be learned in the school and out of school context with the real-world authentic environmental problems trying to propose real-life contributions by examining different alternative options. Students gain integrated learning experiences approaching experiential learning which focuses on the idea that the best ways to learn things is by actually having experiences. Dealing with authentic real life environmental problems need the interaction with different stakeholders in the community, with various interests, tasks and priorities. Therefore, students experience an inter-generational (inter-aged) learning including students and peers along with adults from the various stakeholders (e.g., researchers, scientists, experts, NGOs, economic factors, enterprises and social factors).

EEC also encompass that learning integrates community service activities in the attempt to serve for a contribution to find a possible solution to the environmental problem under study. Learning in EEC complement service within the community and enables students to reflect upon and address local and national environmental problems. This cannot be done without active civic participation. EEC would like

learners to be involved as actively in the learning process as possible. Civic participation and active engagement with authentic real-life socio-environmental problems includes an intentional sequence of activities or learning events that will help the learner achieve a specified contribution for the solution of the environmental problem under study. It also asks for a highly hierarchical type of participation including influence, participation in decision making and community involvement. The critical praxis implies elements of critical pedagogy and the ability to critically examine and evaluate the complexities, patterns, and policies that permeate local and global environmental problems. It also includes examining the structural causes of the specific environmental problem which involves critical and emancipatory learning. However, EEC asks to move beyond the local context, aiming also at national and global action with individual and collective actions in private and public spheres, including students' activism. For EEC it is important to examine similar environmental problems in other places, in other countries, even in other continents. It studies the similarities and differences in such cases and examines also cases of inter- and intra-generational injustices in relation to the environmental problem under study at the local, national and global scale (Environmental Justice Pedagogy). EEC, therefore, is aiming at environmental and social change and consequently, is not only an action-oriented education but a change-oriented education attempting an environmental and social transformation for a neutral, green and just transition as a transformative learning. Table 5.1 shows the main characteristics of EEC.

Table 5.1 Characteristics of EEC.

	Characteristics
1.	Comprehensive and integrated learning
2.	Authentic real-life learning
3.	Experiential learning
4.	Inter-generational (inter-aged) learning
5.	Community service learning
6.	Participatory learning
7.	Critical and emancipatory learning
8.	Local, National and Global Action learning
9.	Inter- & Intra-generational justice learning
10.	Students' activism learning
11.	Change oriented learning
12.	Transformative learning

5.3.4 Notions of Education for Environmental Citizenship

It is important to mention that EEC includes the Global notion, the Responsibility notion, the Participative notion, the Democratic notion and the Co-creation notion. Regarding the Global notion, as already mentioned above, EEC includes individual and collective actions at local, national and global level and therefore the global dimension of this type of education is strong and clear. With this approach it prepares the culture of "citizens of the world" who care about both the local level and the national and global level. In this frame, the EEC embraces the dimensions of Global citizenship education as well as cosmopolitanism. As far as the notion of responsibility, we recall the clear reference of the EEC definition for ''…responsible pro-environmental behavior''. This can be matched to the personal behavior of a citizen, where citizens should be honest, responsible and law-abiding members of the community. In addition, the inclusion of inter- & intra- generational justice and the practice of environmental rights and duties entails in addition the second dimension of Young's (2006) two-tiered model of Responsibility. The Participative notion is also crucial, since critical and active engagement and civic participation to address structural causes are included in the definition of the EEC. These references clearly connect to critical pedagogy (Freire, 1987) and transformative education (Mezirow, 1978). The connection with civic participation and the highly hierarchical types of participation is also clear (see Arnstein's ladder of participation, 1969). Of course, a collective participation is included. All of these can be considered as the participative notion of Education for Environmental Citizenship. Regarding the Democratic notion, the ENEC definitions state that environmental citizens should address the structural causes of environmental problems through democratic means. This reference is again very important because it refers to the democratic citizen, to democratic education and clearly to the democratic notion of EEC. Finally, the Co-creation notion has substantial positioning in the EEC Pedagogical Approach (Hadjichambis & Paraskeva-Hadjichambi, 2020) taking into account that it is clearly stated that it is important to search for cases of inter- and intra-injustice. In addition, the decision making on alternative solutions, the inclusion of collective design and ownership refer to the co-creation notion of Environmental Citizenship.

5.3.5 Education for Environmental Citizenship Competences

Based on the reasoning that has been developed so far and based on the EEC Model, it is obvious that in order for the EEC outputs to be achieved, a number of competences are necessary, which have been mentioned scattered throughout the chapter. It is of paramount importance for environmental citizens to have the necessary competences to act individually and collectively as agents for environmental and social change. In addition, competences related to the critical and active engagement and civic participation of citizens are necessary in order for them to be able to act

effectively in civil society through different critical socio-political actions. It is also essential that environmental citizens have the competences to plan, consider alternatives, implement and evaluate individual and collective actions, both individual and collective dimensions, and on local, national and global scales to achieve EEC outputs. Furthermore, environmental citizens need to have the competencies to address environmental transformative justice issues including intra- and inter-generational justice and injustice. These competences relate to addressing unsustainability and the structural causes of environmental problems within democratic means. Competences related to environmental problem solving and preventing as well as restoring environmental degradation are also very important. Social skills (e.g., collaboration, communication, negotiating and resolving conflicts) are also important. In addition, argumentation and decision-making skills, critical thinking, systems thinking, scientific or evidence-based thinking, and creative and empathic thinking (e.g, Schusler et al., 2009; Berkowitz et al., 2005; Mintzes et al., 1998; Schauble, 1996) are needed. Therefore, integrated skills with a coherent body of knowledge and appropriate attitudes should be interwoven for an effective environmental citizen.

5.3.6 Comprehensive and Holistic Approach of Education for Environmental Citizenship

At this point, it is worth briefly presenting a Pedagogical Approach that can promote Education for Environmental Citizenship. The proposed pedagogical approach includes all those elements that are necessary to achieve the outcomes of the EEC Model. Usually, it all starts with a local or a global environmental problem. This problem should be highlighted by the students themselves in order to be motivated to explore it, find solutions and take an active role to solve it. It can also be a problem faced by the community in which the students' school is located or where they live and believe that they can act as environmental citizens to help solve it. A starting point, however, could be one global environmental problem which may have an impact on their community (such as climate change) and thus make students feel the need to contribute as agents of change, and also to give them the opportunity to expand their actions through networking locally or globally.

This Education for Environmental Citizenship Pedagogical Approach can be implemented through six distinct stages: Inquiry, Action Planning, Critical and Active Citizen Participation and Engagement, Networking & Sharing in Scales (local, national, global), Sustain Environmental and Social Change and finally Evaluation & Reflection (Fig. 5.4). The entry point in the implementation of the Pedagogical Approach can be any of the six stages depending on what suits the issue under investigation. Therefore, the 6 stages are not proposed to be implemented in a linear sequence. Also, at each stage some steps are suggested that support the implementation of each stage. Although it is not necessary to implement all steps, it is important to include some actions that fall into each of the 6 proposed stages, as each stage promotes different aspects of environmental citizenship. The combination of the

activities at the various stages, as described below, could fulfil the aims of this Pedagogical Approach to foster Environmental Citizenship.

The **Inquiry** stage includes five steps: Data collection and analysis, Structural causes, Inter- & Intra-generational injustice, Value clarification, and Place-based activities. At this stage, students are asked to collect data and proceed with their analysis, so that they can understand the different dimensions of the problem. Many times, the collection of scientific data could be the starting point for students in order to develop and support their argumentation towards a problem solution. At this stage, students collect and analyze data regarding an environmental problem. They are given the opportunity to examine the structural causes of the environmental problem for example, and may identify behind the problem ineffective environmental laws or ineffective procedures for nature conservation, conflicting interests for a development or prioritization of economic development over environmental protection. Students may also have the opportunity to examine cases of intra- & inter-generational injustice in relation to the environmental problem. For example, students could detect the accumulation of wealth in certain land developers (intra-generational injustice) or the violation of environmental rights and obligations in such a way that future generations will be deprived of certain ecosystem services (inter-generational injustice). The values driving different stakeholders (e.g., developers, ecologists) relevant to the environmental problem are also important for students to understand. For example, what values are hidden behind the positions of the various stakeholders (e.g., developers, students, environmentalists, etc.)? Finally, it is important for students to visit the site in which the problem exists and take part in outdoor and place- based activities in the field.

Planning Actions is another very important stage of the Education for Environmental Citizenship Pedagogical Approach. At this stage, students should be able to plan individual and collective actions in the private and public sector. To achieve this, it would be helpful for students to record the stakeholders' interests in the environmental problem under study. For example, in a local environmental problem, the relevant stakeholders could be developers, environmentalists, students, politicians, the government, the community. In a next step, it may be useful for students to capture and map the stakeholder controversy by elaborating the arguments for or against a proposed solution. By decoding the controversy, students will realize the complexity of the environmental problem studied (e.g., Latour, 2005) and be able

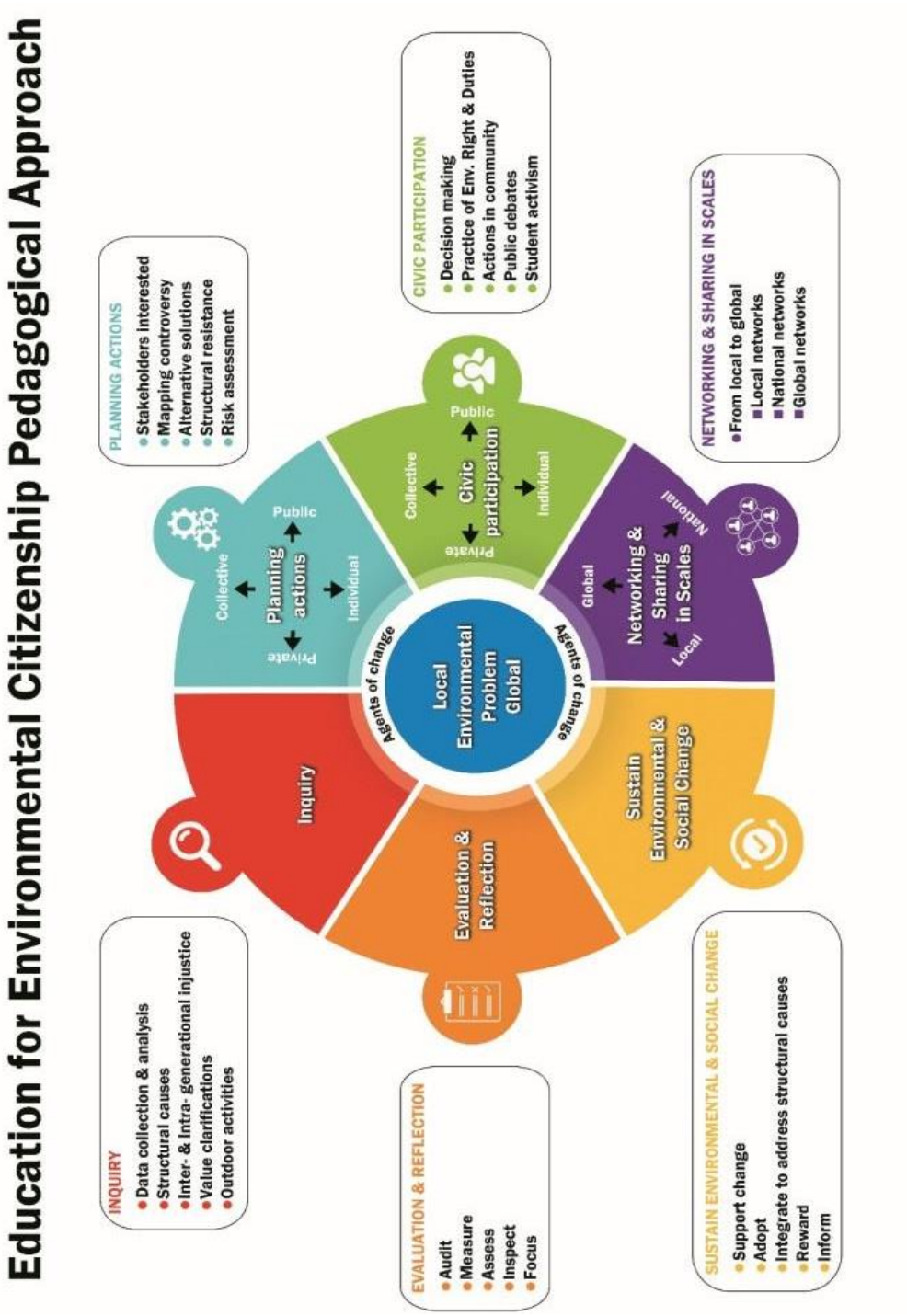

Fig. 5.4 The EEC Pedagogical Approach (Source: Hadjichambis and Paraskeva-Hadjichambi, 2020)

to design solutions that take into account the conflicting interests of the actors involved. Another step in action planning is to consider possible alternatives to the environmental problem studied, documenting the pros and cons of each alternative from a sustainability perspective. In a next step, students can explore the structural resistance that the proposed alternatives could face. Some examples of possible structural resistance that could be identified include resistance from the system, inelastic laws, interference from decision-makers, or financial interests promoting growth at the expense of the environment. Finally, at this stage, students could assess the risks from the planned actions. It would be useful for students to anticipate the risks, when planning activities, so that they will be ready to handle potential risks. An example of risk could be a potential disruption and confrontation in the community with accusations on a personal and collective level.

Civic participation is a crucial stage for the implementation of the suggested pedagogical approach. The first and most important aspect of citizen participation is their active involvement in decision-making (Schulz et al., 2016). In this step, students should explore the alternatives they identified in the previous stage and make their decision about the best solution (Paraskeva-Hadjichambi et al., 2015). In this step students can share their decision(s) and suggestions about the optimum alternative with scientists, environmental organizations, politicians and other stakeholders. Another step at this stage is the exercise of environmental rights and obligations. Examples of such rights and duties may include free access to environmental data and information, the right of public participation and consultation, public access to justice, the need for an environmental impact assessment and the environmental assessment documentation strategy. The next step is to implement actions in the community, including individual and collective actions in the private and public sectors. Students could participate in or make a contribution to an environmental campaign, act as volunteers, write an article in a local newspaper, or participate in radio and television broadcasts regarding the environmental issue and the suggested solutions. Organizing or participating in a public debate could be another possible step. Students' participation in public discussions, in addition to the knowledge they will gain through argumentation, can help them develop important communication skills and active participation in the community (Hadjichambis et al., 2018; Owens et al., 2017; Gregory et al., 2005). Finally, the support for students' activism is also important. Informing campaigns for their families, their peers and the general public, organizing and participating in protests or demonstrations can give opportunities for students to practice different forms of civic participation that could equip students with several competences such as knowledge, skills, self-efficacy, self-esteem, and socio-political empowerment (Baptista et al., 2018; Marques and Reis, 2017; Schusler and Krasny, 2015; Simonneaux, 2007). In addition, activism has been proven to be beneficial for environmental and social transformation (Bencze and Carter, 2011; Shor & Freire, 1987).

Networking & Sharing in Scales is another stage that is included in the of EEC pedagogical approach. Students can maximize their impact by organizing local

networks, involving other classmates, experts, people who could voluntarily support their actions, politicians, and even activists, who struggle for the protection of the environment. Thus, students can influence decisions in their community and be a lever of pressure on local communities to realize the importance of solving the specific environmental problem, and also highlight the importance of the precautionary principle in order to avoid creating other similar problems. Students can also shift the discussion and effort to solve this environmental problem nationwide, by developing a global network of students, scientists, volunteers, supporters, activists, politicians and others. Connecting with national environmental NGOs is also important in this step. Although very ambitious, students can also inform the global community about the environmental problem they are studying. They can try to create global action networks by mobilizing students, scientists, volunteers, advocates, activists and politicians in other countries in a global action. Connecting with international NGOs is extremely important. The recent global climate change movement (e.g. FFF - Fridays for Future, a global weekly student activism day) has shown that this effort is not utopian. Social media, social networks, blogs and other recent information technology applications can have a major impact on such efforts (Gerbaudo, 2018).

Sustain Environmental & Social Change cannot be considered as a less important stage of the Pedagogical Approach to Environmental Citizenship. Through this stage, students attempt to make complementary efforts to sustain environmental and social change. Students are given the opportunity to support and improve on previous actions. For example, they can keep discussing the issue for an extra period of time until it is resolved. The topic can be presented in the news and students may adopt new support measures and actions. Another important step at this stage is the integration of additional actions to address structural causes in other areas and at other levels. In this context, students can inform by letter other competent bodies. For example, they can send a formal letter to parliament or to the Minister for the Environment stating an environmental policy deficit. This can be a lack of existing environmental legislation, a lack of enforcement of environmental legislation, a lack of environmental structures and infrastructure or even a lack of environmental "culture". In another step, students could reward those who helped with their actions (e.g., students, volunteers, supporters) by sending, for example, a thank you letter. Finally, they can inform the public about their success and disseminate successful actions, so that it is understood that such actions make sense and have the power to resolve environmental issues.

Evaluation & reflection is an integral part of EEC pedagogical approach. As in any procedure, students should evaluate the success of the several actions implemented (e.g., demonstrations, official letters). They can collaborate with their teachers to create research tools to measure different competencies (e.g., knowledge of students before and after the intervention, attitudes of students before and after, values of stakeholders or the community, skills and abilities). Students can also check the hidden dimensions of the procedures and steps of the applied approach. Finally, students can identify the pros and cons from the implementation of the approach and

use their experience to improve the disadvantages of the process followed of resolving an environmental issue in subsequent efforts.

In conclusion, this EEC pedagogical approach is one of the possible venues for EEC (Hadjichambis et al., 2020) and empirical studies on the implementation of the pedagogical approach can shed light on its effectiveness to foster EEC. Unpublished results from empirical studies implementing the EEC pedagogical approach indicate promising outputs in fostering EEC. However, those results are beyond the scope of this chapter.

5.4 A Case Study from Cyprus – Empirical data

A first attempt to apply the EEC pedagogical approach was implemented in the Cyprus context regarding the development of a Casino Resort nearby a protected wetland (Hadjichambis et al., 2022). This learning intervention was designed based on the EEC pedagogical approach and was implemented with 10th grade biology students (15–16 years old). The learning intervention was implemented as a project embedded in Biology lessons with a duration of 4 months. Students were given the opportunity to participate in several activities related to the 6 stages (and several steps) of the EEC Pedagogical Approach.

A sample of 50 students participated comprised of 29 girls (58%) and 21 boys (42%), from 2 classrooms. Students were of mixed academic ability according to the national educational practices. Each classroom included students whose cognitive abilities ranged from high-average to low-average, as well as some highly-gifted students. The Environmental Citizenship Questionnaire (ECQ) (Hadjichambis & Paraskeva-Hadjichambi, 2020) was employed for data collection and applied before (pre-) and after (post-) the learning intervention. The ECQ is composed by nine (9) closed-ended questions including in total 76 items.

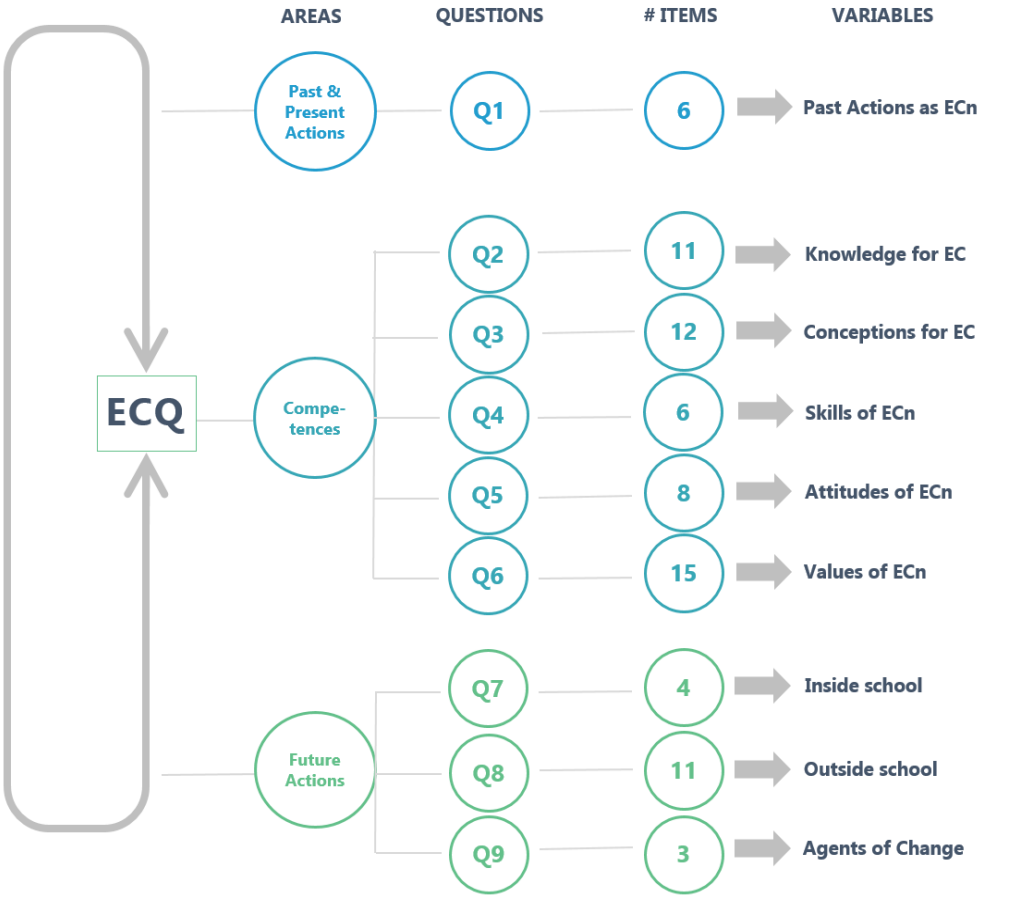

Fig. 5.5 The ECQ structure (Source: Hadjichambis and Paraskeva-Hadjichambi, 2020).

This research tool addresses competences associated with EC (Fig. 5.5) in the cognitive (knowledge, conceptions, and skills) and affective (attitudes, values) dimensions and engagement in actions associated with EC in both private and public spheres currently and with a future-oriented perspective (likeliness of involvement in the future).

Preliminary results revealed that the EEC Pedagogical Approach can significantly contribute to the empowerment of students into active environmental citizens. According to the results of the pre-test the majority of the students had scarcely been involved in activities with environmental organizations or groups outside school, while at school were not given many opportunities to become familiar with ways of preventing or solving environmental problems, practicing environmental rights and duties or actively participating in society. Those parameters were considerably improved in the post-test. In addition, after their involvement in the learning intervention, the development of students' skills as environmental citizens was found to be statistically significant.

The paired t-test (for two dependent samples), revealed a statistically significant difference in all questions before and after the educational intervention. For example, the Difference in Mean scores (Post-Pre) in Past/Present Actions as Environmental Citizens was found 0.88 (SD: 0.42, t: 14.75, p<0,001***). In addition, the Difference in Mean scores (Post-Pre) regarding students' skills as Environmental Citizens was found 0.34 (SD: 0.42, t: 5.75, p<0,001***).

A study from Telešienė et al., (2021) revealed that the ECQ questionnaire can be a reliable tool for measuring Environmental Citizenship. In addition, from the first-time implementation of this new tool in the context of higher education and by exploring the validity of this tool in different contexts, the ECQ instrument proved that it can be used in diverse educational contexts and with diverse ages to tap the environmental citizenship of learners in the context of educational interventions (Telešienė et al., 2021). More empirical studies could shed light on the effectiveness of the EEC Pedagogical Approach in promoting Environmental Citizenship. Future empirical studies should also be undertaken investigating the impact of professional development and teacher education initiatives not only on teachers' perceptions on environmental citizenship but also on how these perceptions are reflected in teaching practices, within the school classrooms, in the framework of EEC (Georgiou et al., 2021).

5.5 Conclusions

Education for Environmental Citizenship could enrich biological curricula with an innovative, integrated and holistic perspective combining knowledge, skills, values and beliefs, attitudes, and behaviors with individual and collective environmental actions in private and public spheres as previously described. Such a perspective removes the walls that isolate school from society and science and allows for the elaboration of important partnerships between school, science and society. This chapter strengthens the significance of the integration of Education for Environmental Citizenship in dealing with complex socio-scientific environmental and problems and introduces expanded ways of thinking as it proposes the establishment of Education for Environmental Citizenship as a distinct, integrated and holistic educational field with its own aims and primary tasks. It includes Environmental Justice Pedagogy incorporating the practice of environmental rights and duties, the promotion of inter-and intra-generational justice and addressing cases of injustice. In addition, it includes Critical and Civic Participation Pedagogy incorporating civic participation, critical and active engagement and addressing structural causes of environmental problems. Finally, it includes Sustainability and Nature Connectedness Pedagogy incorporating sustainability and sustainable development goals as well as the development of a healthy relationship with nature. Conclusively, the EEC pedagogical approach is a promising avenue that could fulfill the imperative need for Education for Environmental Citizenship in biology education.

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Chapter 6
Contribution of conservation biology research to the establishment and effective management of the Natura 2000 Network in Cyprus

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Cite this chapter:

Kadis, C., Andreou, M., Kounnamas, C., Eliades, N. G., Mammides, C., Kouzali, I., Christodoulou, C. S., Hadjichambis, A. Ch. and Georghiou, K. (2025). Contribution of conservation biology research to the establishment and effective management of the Natura 2000 Network in Cyprus. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 141-184). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

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Contribution of conservation biology research to the establishment and effective management of the Natura 2000 Network in Cyprus

Costas Kadis[1], Marios Andreou[1], Constantinos Kounnamas[1], Nicolas-George Eliades[1], Christos Mammides[1], Iro Kouzali[1], Charalambos S. Christodoulou[2], Andreas Ch. Hadjichambis[4,5] and Kyriacos Georghiou[3]

1: Nature Conservation Unit, Frederick University, 7, Yianni Frederickou Street, Pallouriotissa, 1036 Nicosia, Cyprus.
2: Department of Forests, Ministry of Agriculture, Rural Development and Environment of Cyprus, Louki Akrita 26, 1414 Nicosia, Cyprus.
3: Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, Panepistimiopolis, Athens 15784, Greece.
4: Cyprus Center for Environmental Research and Education (CYCERE), Agiou Andreou 306, P.O. 56091, 3304 Limassol, Cyprus.
5: Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus.

Keywords: Natura 2000 network, LIFE program, Special Areas of Conservation, Special Protection Areas, conservation projects, *in situ* conservation, *ex situ* conservation

Chapter Abstract: This chapter presents the main outcomes of more than 20 conservation projects that have been carried out by the Department of Botany of the National and Kapodistrian University of Athens and the Nature Conservation Unit of Frederick University, in close collaboration with the competent authorities of Cyprus. These projects have been co-funded by the European Commission and the Government of Cyprus and have contributed towards (i) accumulating scientific knowledge on the population size, the geographical distribution, the conservation status, the habitat characteristics and the reproductive biology of targeted species, as well as the characteristics and vulnerability of Annex I, Directive 92/43/EEC, habitat types, (ii) developing and implementing action, management and monitoring plans focusing on species and habitats conservation, and (iii) implementing species and habitat specific in situ and ex situ conservation measures. The results of these projects have played a catalytic role in the delineation, operation, and effective management of the Natura 2000 network in Cyprus, while also contributing to raising public awareness about the role of the Natura 2000 Network and, more generally, about biodiversity conservation.

Acknowledgments
Special thanks are extended to the competent authorities of the Republic of Cyprus, particularly to the Department of Forests and the Department of Environment of the Ministry of Agriculture, Rural Development and Environment, as well as to all individuals who contributed to the implementation of the projects presented in this chapter. Moreover, thanks are expressed to the European Commission, the Research and Innovation Foundation, the United Nations Development Program, and other funding bodies, since without their co-financing the projects would not have been possible to implement.

6.1 Introduction

The Natura 2000 Network is the largest network of protected areas in the world and serves as the cornerstone of the European policy for environmental and biodiversity protection. It covers 18.6% of the European territory, of which 62.92% (767,885 km²) consists of terrestrial areas and the remaining 37.08% (452,502 km²) of marine Natura 2000 areas. The establishment and operation of the Network are defined by the Directives 92/43/EEC and 2009/147/EC, which all Member States are required to adopt by incorporating their provisions into national law (European Commission 2024).

The preparatory work for the creation of the Natura 2000 Network in Cyprus began even before the Republic of Cyprus joined the European Union in 2004. Specifically, through the LIFE project – Third Countries Special Areas of Conservation (Directive 92/43/EEC) in Cyprus, the provisions of the Directive were adopted to propose specific areas for inclusion in the Network, as well as specific habitat types and species for inclusion in the Annexes of the two Directives. The project was implemented under the initiative of the Department of Botany, of the Faculty of Biology, of the National and Kapodistrian University of Athens, in close collaboration with the relevant government services of Cyprus. These agencies collaborated for the effective operation of the Natura 2000 Network. With the establishment of the Nature Conservation Unit (NCU) at Frederick University in 2005, this collaboration expanded and strengthened, becoming a point of reference today, both for the operation of the Natura 2000 Network in Cyprus and for the conservation of the island's biodiversity. The staff of the two universities, always in cooperation with the competent authorities, have implemented more than 20 research projects to date, directly and indirectly related to the smooth functioning of the Network and the conservation of species and habitats, while also fulfilling the obligations of the Republic of Cyprus under EU law. Nowadays, the Natura 2000 Network in Cyprus consists of 39 Special Areas of Conservation (SACs), 4 Sites of Community Importance (SCIs), and 33 Special Protection Areas (SPAs), covering a total area of 10378.57 km^2, both in terrestrial and marine environments. In these areas, 47 habitat types, 49 *taxa* of flora and fauna, and 129 bird species are found, of which, five habitat types, two mammals, three reptiles, one invertebrate, six birds, and 17 plant species (Christodoulou 2003) are new additions to the Annexes of Directives 92/43/EEC and 2009/147/EC (Delipetrou and Christodoulou 2004, Department of Environment 2024).

This chapter provides a brief overview of these projects, specifically focusing on their goals and achievements. These projects have been co-financed by the European Commission and the Republic of Cyprus and have contributed towards the:

- Accumulation knowledge regarding the population size, geographic distribution, conservation status, habitat, and reproductive biology of species listed in Annexes II and IV of the Habitats Directive, as well as the characteristic species and threats faced by habitat types of Annex I of the same Directive.
- Development and implementing action, management, and monitoring plans focused on the conservation of species and habitats.
- Implementation targeted conservation measures, both *in situ* and *ex situ*, for species and habitat types.
- Promotion public awareness regarding biodiversity conservation and the significance of the Natura 2000 Network.

6.2 Research projects related to biodiversity conservation and the operation of the Natura 2000 Network in Cyprus.

In the text below, 20 research projects are briefly described, co-funded by the European Commission through the LIFE Program (12 projects), the Research and Innovation Foundation (formerly the Research Promotion Foundation) (6 projects), or other funding sources (2 projects).

6.2.1 LIFE projects

6.2.1.1 Life Third Countries

The project entitled 'Special Areas of Conservation (Directive 92/43/EEC) in Cyprus' (LIFETCY98/CY/172) was implemented within the framework of the European Union's (EU) Life Third Countries program. The total project budget was 350,000 European Currency Unit (ECU), of which 175,000 ECU (50% of its total eligible budget) were co-funded by EU. The project aimed at providing the information needed for the establishment of a network of conservation zones in Cyprus, within the European Ecological Network 'Natura 2000' (Directive 92/43/EEC). Specifically, the project focused on the establishment of: (a) a national list of SACs; (b) a database containing the Standard Data Forms for the SACs and (c) a national archive of ecological data in Cyprus. The project was implemented by the Environment

Service (Coordinating Beneficiary), the Department of Forests, the National and Kapodistrian University of Athens and the Enalion Environmental Management Centre LTD, from April 1999 to March 2001.

The main achievements of the project were:

- 38 sites were proposed for inclusion in the Sites of Community Importance (terrestrial and marine) and 8 sites as Special Protection Areas.
- 52 habitat types were mapped, of which five were new additions to Annex I of the Habitats Directive, while for four other habitats, a modification was made to the definitions of the existing habitats of Annex I, so that their description corresponds to the particularities they present in Cyprus (Delipetrou and Christodoulou 2004).
- 109 bird species were recorded, of which 6 are new additions to Annex I of the Birds Directive.
- Nine mammals (two are new additions), 5 reptiles (three are new additions), 1 fish and 2 invertebrates (one is a new addition) of Annex II of the Habitats Directive were recorded.
- 17 (16 endemic and 1 indigenous) plant species and subspecies, out of which 8 as priority species, were proposed for inclusion in Annex II of Habitats Directive (Χριστοδούλου 2003).

6.2.1.2 LIFE – COMANACY

The project entitled 'Conservation management in Natura 2000 sites of Cyprus' (LIFE04 NAT/CY/000013) was implemented within the framework of European Union's (EU) LIFE program. The total project budget was 2,551,277 euro, of which 1,530,766 euro (60% of its total eligible budget) were co-funded by EU. The project was the first applied attempt of scientific management of five Natura 2000 sites in Cyprus: Kavo Gkreko - CY3000005, Koilada Diarizou – CY4000003, Vouni Panagias - CY4000004, Troodos National Forest Park - CY5000004 and Alykes Larnakas - CY6000002. The main objective of the project was the implementation of immediate actions in order to secure a favourable conservation status for the natural habitats and wild species in the sites, setting the standards for the management of Natura 2000 Network in Cyprus. The project was implemented by the Environment Service (Coordinating Beneficiary), the Department of Forests, the Department of Fisheries and Marine Research, the Game & Fauna Service, the National and Kapodistrian University of Athens, the Greek Biotope/Wetland Centre, the OIKOS- Nature Management Ltd and the ATLANTIS Consulting Cyprus Ltd, from November 2004 to November 2008. The targeted species and habitat types are shown in Table 6.1.

The main achievements of the project were:

- Elaboration of Management Plans for the five Natura 2000 sites.
- Elaboration of Monitoring Plans for targeted habitat types and species.
- Monitoring of targeted priority habitats and species according to the Monitoring Plans.
- Restoration and expansion of *Quercus infectoria* subsp. *veneris* woodland (93A0 habitat type) at Vouni Panagias.
- Enhancement of the population of **Scilla morrisii* at Vouni Panagias.
- Improvement of prey and water availability for *Hieraaetus fasciatus*.
- Pilot management of *Pinus nigra* subsp. *pallasiana* forest (9536*) in Troodos National Forest Park (TNFP).
- Protection of Peat grasslands (6460* habitat type) in TNFP.
- Protection of the habitat of Serpentinophilous grasslands (62B0* habitat type) in TNFP.
- Protection of the populations of **Scilla lochiae* and **Pinguicula crystallina* and enhancement of the population of *Arabis kennedyae** in TNFP.
- Enhancement of *Alnus orientalis* riparian woodland (92C0 habitat type) in Koilada Diarizou.
- Rehabilitation of *Ziziphus lotus* mattoral (5220* habiat type) in Kavo Gkreko.
- Protection of Vernal Pools (3170* habitat type) in Kavo Gkreko.
- Installation of an anchoring system for vessels in Kavo Gkreko to protect 1120* habitat type (*Posidonia* beds) (Georghiou et al. 2008).

Table 6.1. Actions and results for the targeted habitat types and species of LIFE-COMANACY project. (Source: Georghiou et al. 2008).

Targeted habitats and species of the project

Habitats	Sites	Results
Posidonia beds (1120*)	Kavo Gkreco	Monitoring, pressure/threat alleviation (anchoring)
Vernal pools (5220*)	Kavo Gkreco	Monitoring, protection (fencing, signposting)
Mattoral with *Ziziphus* (5220*)	Kavo Gkreco	Monitoring, restoration (0.84 ha increase of habitat cover with 3 new locations)
Mediterranean riparian forests with *Alnus orientalis* (92C0)	Diarizou valley	Monitoring, restoration (12,5 ha increase of habitat cover by protection of natural regeneration)
Forest stands with *Quercus infectoria* (93A0)	Vouni Panagias	Monitoring, restoration (12 ha increase of habitat cover with 1 new location)
Serpentinophilous grasslands (62B0*)	Troodos NFP	Monitoring, protection (fencing, signposting)
Peat grasslands (6460) in Troodos	Troodos NFP	Monitoring, protection (pressure/threat alleviation by delimitation, signposting, fencing, removal of picnic site)
Pinus nigra forest (9536*)	Troodos NFP	Monitoring, conservation (study and regeneration enhancement)
Annual vegetation of mud and sand (1310)	Alyki Larnakas	Protection, restoration (pressure/threat alleviation by removal of acacia trees and garbage, restriction of vehicle passage, maintenance of channels, public awareness/information actions)
Mediterranean salt meadows (1410)	Alyki Larnakas	
Mediterranean halophilous scrub (1420)	Alyki Larnakas	

Plants	Sites	Results
*Scilla morrisii**	Vouni Panagias	Monitoring, protection by fencing, restoration (3 new locations)
*Arabis kennedyae**	NFP Troodos	Monitoring
*Chionodoxa lochiae**	NFP Troodos	Monitoring, protection by fencing
*Pinguicula crystallina**	NFP Troodos	Monitoring, protection by fencing

Reptile	Sites	Results
*Coluber cypriensis**	NFP Troodos	Monitoring

Birds	Sites	Results
*Aquila heliaca**	Troodos NFP	Monitoring, habitat improvement in Diarizos valley (maintainance of springs, troughs, artificial lakes), birdwatching hides construction at Alyki Larnakas, public information and awareness actions about the impacts of baits and pesticides.
*Aythya nyroca**	Alyki Larnakas	
*Branta ruficollis**	Alyki Larnakas	
Buteo rufinus	Vouni Panagias, Troodos NFP	
Certhia brachydactyla dorotheae	Troodos NFP, Kavo Gkreko	
Charadrius alexandrinus	Vouni Panagias, Alyki Larnakas	
Circus aeruginosus	Vouni Panagias, Troodos NFP, Diarizos, Alyki Larnakas	
Circus aeruginosus	Alyki Larnakas	
Circus cyaneus	Vouni Panagias, Troodos NFP, Kavo Gkreko, Alyki Larnakas	
Circus macrourus	Vouni Panagias, Troodos NFP, Kavo Gkreko, Alyki Larnakas	
Egretta alba	Alyki Larnakas	
Egretta garzetta	Alyki Larnakas, Kavo Gkreko	
Emberiza caesia	Vouni Panagias, Kavo Gkreko	
*Falco eleonorae**	Vouni Panagias, Troodos NFP	
Falco peregrinus	Vouni Panagias, Troodos NFP	
Garrulus glandarius glaszneri	Vouni Panagias, Troodos NFP	
Gelochelidon nilotica	Alyki Larnakas	
Gyps fulvus	Vouni Panagias, Troodos NFP	
*Hieraaetus fasciatus**	Vouni Panagias, Troodos NFP	
Himantopus himantopus	Alyki Larnakas	
Ixobrychus minutus	Alyki Larnakas	
Lanius minor	Vouni Panagias, Kavo Gkreko	
Lanius nubicus	Vouni Panagias, Diarizos	
Loxia curvirostra guillemardi	Vouni Panagias, Troodos NFP	
Nycticorax nyxticorax	Alyki Larnakas	
Oenanthe cypriaca	Vouni Panagias, Troodos NFP, Kavo Gkreko, Diarizos	
*Oxyoura leucocephala**	Alyki Larnakas	
Parus ater cypriotes	Vouni Panagias, Troodos NFP	
Pernis apivorous	Vouni Panagias	
Philomachus pugnax	Alyki Larnakas	
Phoenicopterus ruber	Alyki Larnakas	
Platalea leucorodia	Alyki Larnakas	
Plegadis falcinellus	Alyki Larnakas	
Recurvirostra avocetta	Alyki Larnakas	
Sylvia melanothorax	Vouni Panagias, Troodos NFP, Kavo Gkreko, Diarizos	
Sylvia rueppelli	Vouni Panagias, Kavo Gkreko	
Tringa glareola	Alyki Larnakas	

6.2.1.3 PLANT-NET CY

The project entitled 'Establishment of Micro-Reserves Network for Plants in Cyprus for the Conservation of Priority Species and Habitats' (LIFE08 NAT/CY/000453), had a total budget of 1,550,297 euro, out of which 1,070,265 euro (69% of its total eligible budget) were co-funded by the EU. The project's main aim was the improvement of the conservation status of four flora *taxa* (**Arabis kennedyae*, **Astragalus macrocarpus* subsp. *lefkarensis*, **Centaurea akamantis* and **Ophrys kotschyi*) and two habitat types (9590* Forests with *Cedrus brevifolia* (*Cedrocetum brevifoliae*) and 9390* Scrub and forest stands of *Quercus alnifolia*), through the implementation of the Plant Micro-Reserve (PMR) approach. The project was implemented by the Department of Environment (Coordinating Beneficiary), the Department of Forests, the Nature Conservation Unit of Frederick University and the National and Kapodistrian University of Athens, the Federation of Environmental Organizations of Cyprus and the United Nations Development Programme (UNDP-ACT), from January 2010 to July 2013 (Kadis et al. 2013).

The Plant Micro-Reserve (PMR) approach was developed in the 1990s in Spain, aiming to protect selected portions of populations of endemic, rare, and threatened species through the establishment of a continuous monitoring system and the implementation of targeted measures for their conservation within an area of approximately 20 ha (Laguna 2001, Laguna et al. 2004, 2013, Fos et al. 2014). Over the years, the PMR approach has expanded as a conservation model for rare and endemic flora species beyond Spain (Saldãna et al. 2013, Rubio 2013, Carrión et al. 2013) and to other countries such as Slovenia (Sovinc and Lipej 2013), Bulgaria (Natcheva et al. 2013, Bancheva et al. 2014), Cyprus (Kadis et al. 2013, Eliades et al. 2021), Greece (Thanos et al. 2013), Italy (Troia 2013), and Lebanon (Laguna et al. 2018). Over the years, the implementation of the PMR approach has evolved, transitioning from the initial trend of establishing and managing a large network of PMRs to developing a network with a small number of PMRs where intensive monitoring and conservation practices for threatened species are implemented (see Eliades et al. 2021).

The main achievements of the project were:

- Institutionalization of PMRs as areas with special protection status, under the Forest Law ((L. 25(I)/2012)).
- Establishment of a PMR in four sites of the Natura 2000 network: (i) Mitsero Area (CY2000003) for the species of **O. kotschyi* (PMR 1), (ii) Koilada Kedron - Kampos Area (CY2000008)[1] for habitat 9390* Scrub and forest stands of *Q. alnifolia*

[1] The Natura 2000 site "Koilada Kedron - Kampos" (CY2000008) together with the sites "Periochi Agiatis" (CY4000011), "Stavros tis Psokas - Karkavas" (CY4000012) and "Periochi Platy" (CY20000007) were recently included in a large, consolidated site named "Paphos Forest" (CY2000016).

(PMR 2) and for habitat 9590* Forests with *C. brevifolia* (*Cedrosetum brevifoliae*) and the species of **A. Kennedyae* (PMR 3), (iii) Akamas Peninsula (CY4000010) for the species of **C. akamantis* (PMR 4), and (iv) Asgata Area (CY5000007) for the plant **A. macrocarpus* subsp. *lefkarensis* (PMR 5).

- Elaboration and implementation of Monitoring Plans and Management Plans.
- Protection, through individual fencing, of individuals or young plants of target species.
- Implementation of an insect monitoring system that preys on the seeds of the target species **A. macrocarpus* subsp. *lefkarensis*, and adoption of mild management measures for insects (predators) by isolating them with nets during the pollination period.
- Artificial pollination for the target species **O. kotschyi* for four consecutive years (Figure 6.1).
- Small-scale relief modification, with hand tools (area 1-3 m^2), preventing soil erosion and achieving the creation of "benches" for the installation of individuals of **A. macrocarpus* subsp. *lefkarensis*.
- Removal of competitive vegetation and creation of clearings for the installation of individuals of **A. macrocarpus* subsp. *lefkarensis* and **C. akamantis*.
- Removal of flammable biomass for two years to reduce the risk of fire and habitat destruction of the species **O. kotschyi* within PMR 1.
- Moderate water provision during the summer period and at intervals with dry conditions for selected individuals of the target species within PMR 3 & PMR 4 for periods of three and two years, respectively.
- Collection and storage of seeds (*ex situ* conservation) for the target species **O. kotschyi*, **A. kennedyae*, *C. brevifolia*, **C. akamantis* and **A. macrocarpus* subsp. *lefkarensis*, at the Seed Banks of the Agricultural Research Institute and the Nature Conservation Unit of Frederick University.
- Seed dispersal and planting of seedlings, of the target species and of the characteristic species of the target habitat types (*C. brevifolia, Q. alnifolia*), to enrich their population and enhance regeneration.

The PLANT-NET CY project, competing with a multitude of other European projects, was distinguished as one of the 13 "Best LIFE Nature projects" in 2013, in recognition of the efforts made to protect and preserve the target species and habitats.

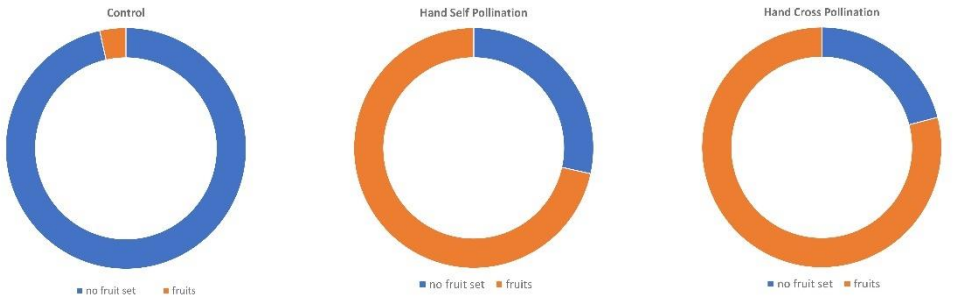

Fig. 6.1 Hand pollination of *Ophrys kotschyi*. (Source: Eliades et al. 2021).

6.2.1.4 ICOSTACY

The project entitled 'Improving the Conservation Status of Fauna Species in Cyprus: from the Restoration of Species Microhabitats to Landscape Coherence' had a total budget of 1,241,007.00 euro and 50% of its total eligible budget, was co-funded by EU. The ICOSTACY project aimed to improve the conservation status of 20 selected fauna species in Cyprus, which are divided into 15 species of bats, three species of reptiles, and two species of invertebrates, as well as their habitats, within the Natura 2000 network areas where they occur. All selected species are included in Annexes II and IV of Directive 92/43/EEC. Specifically, these are: *Hierophis cypriensis*, *Natrix natrix cypriaca*, *Euplagia quadripunctaria*, *Rousettus aegyptiacus*, *Rhinolophus hipposideros*, *Rhinolophus ferrumequinum*, *Rhinolophus euryale*, *Rhinolophus mehelyi*, *Myotis blythii*, *Myotis capacinii*, *Tadarida teniotis*, *Mauremys rivulata* and *Propomacrus cypriacus*. The project's partners were the Department of Environment (Coordinating Beneficiary), the Department of Forests, the Department of Fisheries and Marine Research, the Natural History Museum of Crete (University of Crete), the OIKOS Ltd - Environmental Management and ATEPE - Ecosystem Management, and lasted from October 2010 to March 2014. The project proceeded with the implementation of specific protection measures within 13 Natura 2000 areas (Fig. 6.2).

The main achievements of the project were:

- Construction of four small ponds, a river barrier, a reservoir, and a water channel to enhance the survival of selected reptile species during dry summers and maintain connectivity between different populations.
- Restoration of 13 old buildings and installation of artificial roosts for bats and bird nests, facilitating safe roosting of bat populations. Additionally, entrances to five abandoned mine shafts were successfully constructed to allow safe access for bats to roosting sites.
- Construction of six dry stone walls and 45 stone piles to create favourable conditions (microhabitats) supporting the biological needs of reptile and invertebrate

species, providing shelters and spreading them in areas lacking natural hiding places.

- Construction of three secure underground passages on the road network within the Troodos National Forest Park and the Alykos Potamos - Agios Sozomenos site to ensure safe movement corridors for reptile species between habitats and prevent the risk of biodiversity loss due to vehicular traffic in ecologically sensitive areas.
- Planting, fencing, and maintenance of 150 fruit-bearing trees in selected locations within the Natura 2000 network to provide supplementary food for Egyptian fruit bats.
- Establishment of a tree group (500 trees per location) with oak, oriental plane, and carob species in four selected locations within the Natura 2000 network, to enhance landscape heterogeneity, thereby promoting the conservation of invertebrate species.
- Management of competitive (invasive) species by collecting and removing individuals of the turtle *Trachemys scripta elegans* from five wetlands where the Cypriot terrapin *Mauremys rivulata* occurs, aiming to reduce competition for food and basking sites.
- Genetic composition study of populations from different sites for reptiles (*Hierophis cypriensis, Natrix natrix cypriaca, Mauremys rivulata*), invertebrates (*Callimorpha quadripunctaria, Propomacrus cypriacus*), and bats (*Plecotus kolombatovici*). The study revealed that no isolated population required additional protection measures (Natural History Museum of Crete 2012).
- Implementation of captive breeding methods for reptiles (*Hierophis cypriensis, Natrix natrix cypriaca, Mauremys rivulata*), followed by the release of young individuals into suitable natural areas, achieving an increase in knowledge about these (ATEPE – Ecosystem Management 2014).

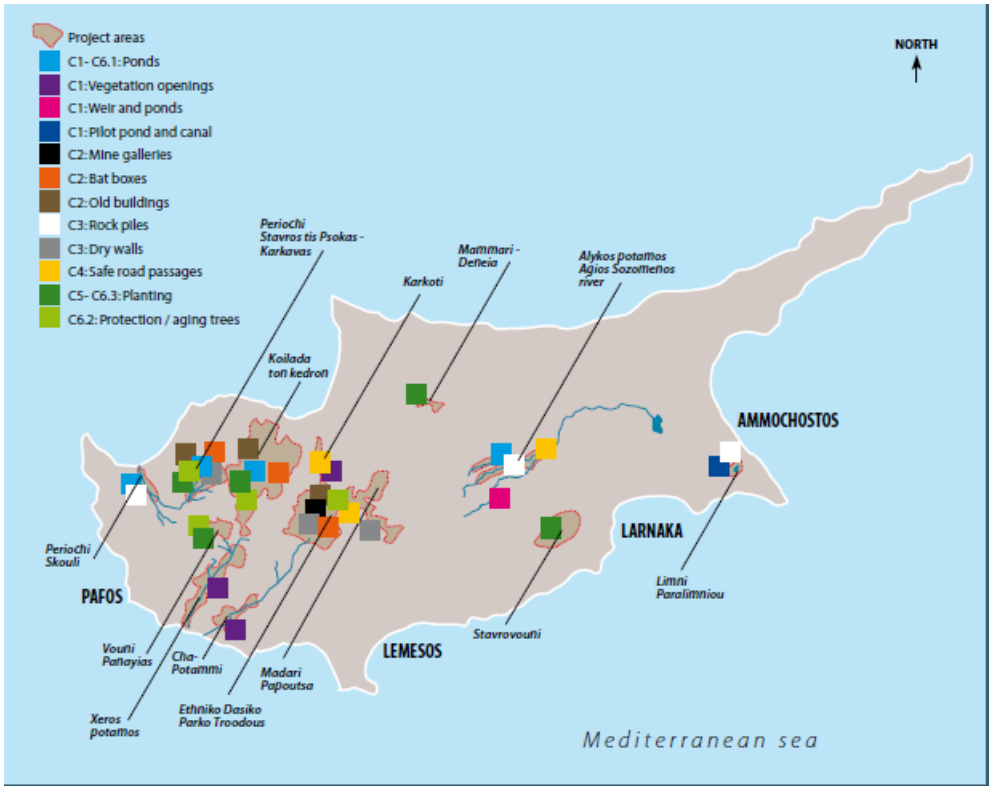

Fig. 6.2 Distribution map of Natura 2000 sites where conservation actions of the ICOSTACY project were implemented (Source: ATEPE – Ecosystem Management 2014).

6.2.1.5 JUNIPERCY

The project entitled 'Improving the conservation status of the priority habitat type 9560* (Endemic forests with *Juniperus* spp.), had a total budget of 1,181,922 euro, of which 886,338 euro (75% of its total eligible budget) were co-funded by the EU. The project's primary objective was to promote and enable the long-term conservation of 'Endemic forests with *Juniperus* spp.' in Cyprus and this was the first project to target this priority habitat in Cyprus. The project was implemented within three Natura 2000 network sites due to the different *Juniperus* sp. that form forests in each site: Madari-Papoutsa (*Juniperus excelsa*), Chersonisos Akama (*Juniperus phoenicea*) and Troodos National Forest Park (*Juniperus oxycedrus and Juniperus foetidissima*). The project's partners were the Department of Forests (Coordinating Beneficiary), the Cyprus Forest Association, the Open University Cyprus, the Nature Conservation Unit of Frederick University and the AKTI Kentro Meletwn k Ereunas. It started in January 2012 and was completed in June 2015.

The main achievements of the project were:

- Mapping of habitat type 9560* in Cyprus: 1,5 ha of forest with *J. excelsa* at Madari, 14,5 ha of pure stands of *J. phoenicea* in Akamas, 263,4 ha of forest with *J. foetidissima* and 1 ha with *J. oxycedrus* (first record) in Troodos.
- Elaboration and implementation of Monitoring Plans and Management Plans.
- Installation of 1,230 m of chain-link fencing in Akamas and 246 m in Troodos.
- Treatment against competitive vegetation within 0.92 ha of habitat in Akamas, 0.35 ha in Madari and 8.5 ha in Troodos.
- Installation of 10 information signs prohibiting lighting of fire, maintenance of fire breaks and creation of a new fire break.
- Implementation of fire prevention measures which benefited approx. 7-9 ha of habitat.
- Installation of 16 notice boards, 20 direction signs and 10 signs with information about the conservation interventions.
- Installation of 25 restriction barriers in Akamas, 30 barriers in Makria Kontarka (Troodos), and 320 barriers in Chionistra area (Troodos).
- Construction of two 90-ton water tanks with adjusted hydrants in Akamas.
- Fencing of 30 *J. phoenicea* individuals to stop grazing.
- Planting of 910 *J. phoenicea* saplings in Akamas, 285 *J. foetidissima* and 328 *J. oxycedrus* saplings in Troodos and 355 *J. excelsa* in Madari to enhance their population.
- Planting of 315 saplings of other species of habitat type 9560* in Troodos, 25 in Akamas and 106 in Madari.
- Collection of 15 kg of seeds (5 kg *J. foetidissima*, 3.35 kg *J. oxycedrus*, 3.35 kg *J. excelsa*, 3 kg *J. phoenicea*) which were stored at Department of Forests' Seed Bank in Amiantos, for the ex-situ conservation of target species (Zomeni 2015).

The JUNIPERCY project was distinguished as 1 of the 6 "Best of the Best LIFE Nature projects" in 2015 (Pedro Silva et al. 2016).

6.2.1.6 LIFE OROKLINI

The project entitled 'Restoration and Management of Oroklini Lake Special Protection Area (SPA) (CY6000010) in Larnaca, Cyprus' had a total project budget of 797,070 euro, of which 398,535 euro (50% of its total eligible budget) were co-funded by EU. The project consisted of both conservation and awareness-raising actions that tackled the pressures threatening this important wetland and its main objective was to bring the Oroklini Lake SPA to a favourable conservation status as defined by the EU Birds and Habitats Directives. The project's partners were the

Game and Fauna Service (Coordinating Beneficiary), the BirdLife Cyprus, the Department of Environment, the Department of Forests and the Voroklini Community Council. It started in January 2012 and was completed in December 2014.

The main achievements of the project were:

- Elaboration of studies which included amongst others a hydrological study, an ichthyological study, a topography study and a study identifying the favourable population size for the Spur-winged Lapwing (*Vanellus spinosus*) and the Black-winged Stilt (*Himantopus himantopus*) that would ensure the long-term maintenance of favourable conservation status for Oroklini Lake (setting of Favourable Reference Values).
- Restoration of an area of 3 ha where a flea-market was illegally operating and create a more suitable nesting habitat for important species.
- Fencing to minimise the disturbance to birds and improvement of nesting conditions.
- Installation of a water level control structure in the lake.
- Creation of six nesting islets surrounded by ditches with water in order to provide suitable nesting and feeding areas, secured from predators.
- Creation of eel passes, marking of powerlines and creation of a pedestrian crossing.
- Removal of Invasive Alien Species of plants and planting local trees and shrubs.
- Preparation of an educational package addressed to 6-12-year-old children that focused on the importance of Oroklini Lake, the threats and the need of protection (http://www.orokliniproject.org/).

6.2.1.7 LIFE RIZOELIA

The project entitled 'Improving the conservation status of the priority habitat types *1520 and *5220 at the Rizoelia National Forest Park' (LIFE12 NAT/CY/000758) had a total project budget of 766,746 euro, of which 574,703 euro (75% of its total eligible budget) were co-funded by EU. The primary aim of the project was to promote and enable the long-term conservation of two priority habitat types within the Natura 2000 site ‘Ethniko Dasiko Parko Rizoelias – CY6000006’: 5220* - Arborescent matorrals with *Ziziphus* and 1520* - Gypsum steppes (*Gypsophiletalia*). The project was implemented by the Department of Forests (Coordinating Beneficiary), the Department of Environment, the Nature Conservation Unit of Frederick University and the Open University of Cyprus, from September 2013 to February 2017.

The main achievements of the project were:

- Detailed habitats mapping within National Forest Park (NFP) of Rizoelia and identification of the distribution of the two targeted habitat types.
- Elaboration and implementation of Monitoring Plans and Management Plans.

- Estimation of subpopulation size of the species *Campanula fastigiata* and *Gypsophila linearifolia* (characteristic species of 1520*) within the Park.
- Protocols on germination, growth and out-planting of the species *Ziziphus lotus, Gypsophila linearifolia* and *Campanula fastigiata* were prepared.
- Demarcation and fencing of selected areas (1.3 km) of the habitat 1520*, in order to reduce trampling and destruction of the characteristic gypsophilous species from the visitors in the Park.
- Removal of the competitive vegetation (mainly Acacias), with environmentally friendly way (total area = 7 ha), with the view to reduce competition between species of the two habitat types, in terms of space, and resources.
- Planting of saplings of the characteristic species of the habitat type 5220* at specific areas within the Park with the view to restore and enhance habitat type 5220*.
- Seed dispersal of the characteristic gypsophilous species of the habitat type 1520* at specific areas in order to enhance habitat type 1520*.
- Creation of 120 m in length of stonewalls to prevent soil erosion nearby or within the re-creation sites of habitat type 5220*.
- Taking measures for the reduction of fire risk, such as the removal of dry vegetation in the perimeter of the park (total area = 8 ha) and the limitation of vehicle access in a length of 3.4 km of dirt roads, in order to limit traffic near the two types of habitat.
- Provision of suitable tools (e.g. Action Plans, Monitoring Plans, etc.) to ensure sustainable management of the Rizoelia NFP in the future.
- Training of the personnel of the Department of Forests on innovative methods for the control of invasive and alien species (Manolaki, Andreou, Christodoulou 2017).

6.2.1.8 LIFE – FOR BIRDS

The project entitled 'Improving lowland forest habitats for Birds in Cyprus' (LIFE13 NAT/CY/000176) had a total project budget of 978,718 euro, of which 489,359 euro (50% of its total eligible budget) were co-funded by EU. The project aimed at: (a) implementing conservation/ management measures that will substantially improve ecological conditions for selected bird species listed in Annex I of the Birds Directive occurring in the Natura 2000 sites: Kavo Gkreko (CY3000005), Periochi Kosiis – Pallourokampou (CY6000009) and Potamos Panagias Stazousas (CY6000007), (b) demonstrating the benefits of adopting a more holistic forest management approach that addresses the needs of birds dwelling in or visiting the forest, and (c) contributing towards enhancement of public awareness on the need to conserve wild birds and combating bird crime within the broader project area. The project was implemented by the Department of Forests (Coordinating Beneficiary), the Cyprus Forest Association, the Nature Conservation Unit of Frederick University and the Game &

Fauna Service, from October 2014 to December 2017.

The main achievements of the project were:

- Detailed census of all bird species living in or passing through the three Special Protection Areas.
- Elaboration of a Management Plan for the site 'Periochi Kosiis – Pallourokampou'.
- Improvement of living conditions (food, water, and nesting sites) for the bird species in the three study areas. Specifically, the following were created/constructed:
  - Five traditional orchards with fruit-bearing trees.
  - Four water tanks.
  - 17 water troughs and 2 rainwater collectors.
  - A small dam in the Avdellero area.
  - 200 artificial nests.
  - 14 dry stone walls, 366 m length in total.
  - 12 fields (4 ha in total) with legumes and cereals.
- Methodical removal of acacia trees on a total area of 5.3 ha.
- Restoration of natural habitat types, covering a total area of 6 ha.
- Taking measures to combat poaching and illegal capture of birds using non-selective means such as nets and limesticks. Specifically:
  - 18 infrared cameras were installed.
  - A patrol by the Game and Fauna Service was established exclusively for the project.
  - 74 road accesses were blocked.
  - 66 poaching violations were reported.
  - An awareness campaign was conducted to inform the public about the negative impacts of illegal bird trapping (Dimitrakopoulos 2017).

6.2.1.9 LIFE-KEDROS

The project entitled 'Holistic management of priority habitat 9590* in the Natura 2000 Network area Kedros Valley-Kambos' had a total budget of 1,413,304 euro, out of which 968,330 euro (68.6% of its total eligible budget) are co-funded by EU. The LIFE-KEDROS project aimed to ensure the medium and long-term conservation of the endemic habitat 9590*, namely the *C. brevifolia* forests, both within and outside their natural limits of distribution. The project was implemented by Department of Forests (Coordinating Beneficiary), the Cyprus Forest Association, and the Nature Conservation Unit of Frederick University, from September 2016 to January 2021 (Eliades 2021).

The main achievements of the project were (Eliades et al. 2021, LIFE-KEDROS 2021):

- Elaboration and implementation of Action Plan for the sustainable management and conservation of habitat type 9590*.
- Forestry interventions, in artificial plantations and locations within the distribution limits of *C. brevifolia* species, in a way that enhances the vitality of *C. brevifolia* individuals by reducing competition and pressure from other forest species (*Pinus brutia* and/or *Quercus alnifolia*) or intraspecific competition. Forestry interventions were carried out in a total area of 200 ha and benefited more than 8500 individuals of *C. brevifolia* (~94% of which were young individuals).
- Restoration and expansion of habitat 9590*, through the enhancement of the structure and density of *C. brevifolia* clusters in areas with low natural regeneration, contributing to the increased density and coherence of the cedar forest. Restoration included plantings in an area of 12.31 ha and dispersal of 15.65 kg of seeds in an area of 8.22 ha. The expansion of the habitat 9590* occurred in an area of 9.37 ha after tree planting and seeding.
- Installation of fire protection infrastructure and implementation of measures for fire prevention and non-expansion of forest fires within habitat 9590*. To achieve this goal, the following were implemented:
  - Strengthening forest patrols by the Department of Forests (June - September).
  - Installation of six warning signs for fire danger.
  - Creation of a new firebreak (300 m).
  - Closure of old forest roads totalling ~32 km in length with the installation of barriers and permanent closure of 13 Km of unused old forest roads through cultivating for enhancing natural vegetation.
  - Implementation of forestry interventions along the forest road network (1 km) to facilitate rapid access for firefighting vehicles.
  - Removal of dry biomass from road edges totalling 8 km in length.
  - Construction of two (2) water tanks, each with a capacity of 90 tons, to supply the Department of Forests motorized vehicles in case of fire.
  - Management of flammable dry biomass through: (i) controlled grazing by domesticated animals, (ii) construction of six (6) watering troughs and three (3) small artificial ponds within the habitat, to attract wild fauna species.
- Enhancement of habitat resilience through the control of biotic threats and improvement of abiotic environment, aiming to stabilize and strengthen the vitality of habitat 9590*. Measures implemented included:
  - Population management of harmful organisms by utilizing natural predators, with the installation of artificial nests to attract bat and owl species (e.g., *Otus cyprius* and *Tyto alba*).
  - Application of the practice of mass collection of harmful bark beetle organisms (*Orthotomicus erosus*) that secondarily attack individuals of *C. brevifolia*, achieving a reduction of the harmful bark beetle population at the sites where it was applied, up to 98% within a period of three years.

- Creation of dry-stone walls totalling 176 m in length, preventing soil erosion at their installation sites and improving soil conditions for around 42 mature *C. brevifolia* trees.
- Installation of gabion wire baskets totalling 156 m in length within the central stream passing through habitat 9590*, to retain streams erode and transport sediment. This action contributed to protecting individuals of *C. brevifolia* from negative impact caused by sediments, as well as regulating water flow within the stream.

- Implementation of a strategy for off-site conservation of the *C. brevifolia* species as a complementary action, by developing specific measures, such as:
  - Establishment of a new nursery with 6500 *C. brevifolia* saplings at the Amiantos mine, as part of the overall mine restoration framework.
  - Preservation of 200 kg of *C. brevifolia* seeds in the Seed Bank of the Department of Forests (LIFE-KEDROS 2021).

The completion of the LIFE-KEDROS project contributed decisively to the integration of multiple ecological and productive functions in the distribution range of habitat 9590* (Lorilla et al. 2023). A simulation study showed that, from the completion of the conservation actions of the project, the multifunctionality of the ecosystem services provided by habitat 9590* is expected to benefit, enhancing the social-ecological value of the area (Lorilla et al. 2023). At the same time the study by Lorilla et al. (2023) indicated that conservation measures over a decade horizon (2037) would work to enhance the area from transitional vegetation with moderate provision of ecosystem services to a denser vegetation state providing ecosystem services associated with high forest properties. The results of the LIFE-KEDROS project highlight the importance of sustainable forest management in terms of maintaining and enhancing the coexistence of multiple ecosystem services (e.g. non-timber forest products, erosion and climate regulation, support of biodiversity storage and recreation), which are considered key ecosystem services of Mediterranean forests (MEA 2005, Noce and Santini 2018).

The LIFE-KEDROS project was qualified as the Best LIFE Project (Best of the Best) in the LIFE Nature category of the European LIFE Awards in 2022 (European Commission 2024). It also won the 2023 Energy Globe National Award.

6.2.1.10 iLIFE-TROODOS

The project entitled "Troodos National Forest Park: Promotion of Natural Values and Ecosystem Services" (LIFE16 GIE/CY/000709) had a total budget of 1,313,826 euro, of which 766,125 euro (60% of the total eligible budget) were co-funded by the EU. The project aimed to raise public awareness about the natural values of Troodos National Forest Park (TNFP) (CY5000004), for which it was included in

the Natura 2000 Network, and the ecosystem services it provides. The project was implemented by the Department of Forests (Coordinating Beneficiary), the Department of Environment, the Nature Conservation Unit of Frederick University, and Contact Advertising Agency Ltd, from October 2017 to September 2020.

The main achievements of the project were:

- Implementation/creation, for the first time in Cyprus, of:
  - An integrated information campaign to promote public awareness about the importance of the natural values and ecosystem services of a Natura 2000 site.
  - Visitor information points (kiosks) in a Natura 2000 site, in Cyprus, which include signs with photographic material and an interactive screen with information for guidance, mobility, and entertainment within the TNFP.
  - Facilities for visually impaired individuals in natural areas, for the purposes of information and access, both at the information points and in recreational areas.
  - Applications for smart devices exclusively for a Natura 2000 site. One application aligned with the information provided on the interactive screens at the information points, while the other was a treasure hunt game with three different routes within the TNFP.
- Identification of the ecosystem services provided by the TNFP (Fig. 6.3), which served as the basis for the awareness campaign (Kounnamas and Andreou 2022).
- Contribution to changing public attitude towards the Natura 2000 Network.
- Organization of a photography competition entitled "Troodos, It's in Our Nature!". The best photos were presented in a photography exhibition that was in a tour for about a year to popular locations in Cyprus.
- Production of a documentary about TNFP, its natural values, and the ecosystem services it provides, with special mention on the villages in the area and their stone-built settlements (Andreou et al. 2020).

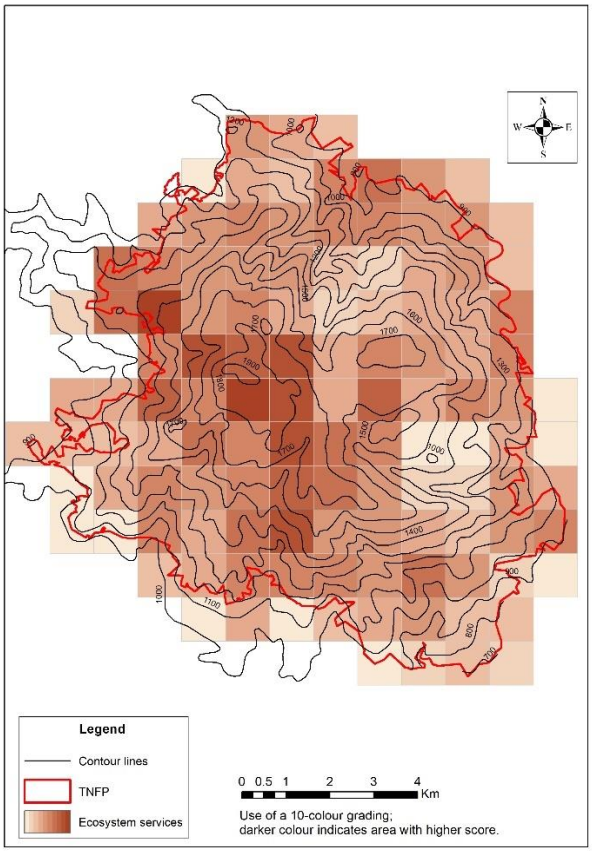

Fig. 6.3 Concentration of Ecosystem Services at Troodos National Forest Park (Source: Kounnamas and Andreou 2022).

6.2.1.11 LIFE CALLIOPE

The project entitled 'Coastal dune hAbitats, subLittoraL sandbanks, marIne reefs: cOnservation, Protection, and thrEats mitigation' (LIFE 17 NAT/IT/000565) had a total project budget of 1,928,505 euro, of which 1,157,103 euro (75% of its total eligible budget) were co-funded by EU. The general aim of the project was to preserve, protect and mitigate the direct and undirect human threats on coastal dunes, sublittoral sandbanks and marine reefs along the Italian coast of central Adriatic and the north-west coast of Cyprus. In these target areas still occur large dune ecosystems and sublittoral marine habitats, but they are threatened by development and tourism activities (which cause trampling and dune habitat degradation), removal of dune vegetation at the beach accesses, nautical tourism and fraud fishing and the lack of

an effective EU protection of natural coastal resources. The project's partners in Italy: Regione Abruzzo (Coordinating Beneficiary), Centro Italiano Ricerche e Studi per la Pesca, Università degli Studi del Molise and in Cyprus: Department of Environment and Nature Conservation Unit of Frederick University. In Cyprus, the project took place in Natura 2000 site 'Periochi Polis – Yialia' (CY4000001). It started in September 2018 and finished in August 2024.

The main achievements of the project in Cyprus were:

- Mapping of habitat types in Natura 200 site 'Periochi Polis – Yialia' and update of the site's Standard Data Form.
- Removal of invasive alien plant species *Acacia saligna* from the beach in order to benefit an area of 12.4 ha (with public funds co-funding).
- Establishment of 130 m of wooden walkways for the minimization of trampling of natural vegetation.
- Installation of 370 m low height fencing (70 m of gabions and 300 m of wooden bollards with ropes) on specific locations in order to limit vehicles access to the beach.
- Collection of seeds and their dispersal, or production of plantlets and their planting at specific locations in order to enhance the continuity of target habitats along the beach and the future stabilization of the soil.
- Implementation of germination experiments and production of a Germination Protocol for the rare plant *Malcolmia nana* var. *glabra*, which is included in the Red Data Book of the Flora of Cyprus as 'Critically Endangered' based on the criteria of IUCN (Kouzali 2023).

6.2.1.12 LIFE IP Physis

The project entitled 'Managing the Natura 2000 network in Cyprus and shaping a sustainable future' (LIFE18 IPE/CY/000006) has a total project budget of 16,999,279 euro, out of which 60% is co-funded by EU. The primary objective of Pandoteira project is to achieve and maintain a favourable conservation status for important species and habitat types in Cyprus, through actions in the whole Natura 2000 network. The project's partners are the Department of Environment (Coordinating Beneficiary), Department of Forests, Game and Fauna Service, Terra Cypria Foundation, Birdlife Cyprus, Open University of Cyprus, Frederick University, Federation of Environmental Organisations of Cyprus, ACC Perivallon kai Kainotomia Limited, I.A.C.O. Environmental & Water Consultants Ltd; Water Consultants Limited, National and Kapodistrian University of Athens, Department of Fisheries and Marine Research, Cyprus University of Technology and AP Marine Environmental Consultancy Ltd. It started in July 2019 and will finish in October 2029.

The project will fill knowledge gaps for species and habitats, improve the govern-

ance of the network, exploit ecosystem services and implement action and management plans for species and habitats. It also aims to positively influence land users, owners, local population and other stakeholders in understanding the importance of the Natura 2000 network and embracing it.

The project includes various actions like:

- Mapping habitat types and species included in Annexes I and II of Directive 92/43/EEC.
- Define a methodological framework for the management of private land on selected Natura 2000 sites.
- Elaboration of 20 Action Plans for species and habitats of Community interest.
- Study the ecological requirements for 7 Annex I bird species of Directive 2009/147/EC.
- Development of a Methodological Framework for the National Ecosystem Assessment.
- Mapping the distribution and action plans for Invasive Alien Species.
- Preparation of a Capacity Building/ Training Action Plan for Better Management of Protected Areas and implementation of the plan.
- Assessment of existing fisheries management measures in new or established Natura 2000 sites and MPAs.
- Development of National Biodiversity Database.
- Holistic approach to the implementation of conservation actions for flora, fauna and habitats.
- Establish management structures appropriate for the areas of the Natura 2000 network outside State Forest Land.
- Ecosystems Services Assessment in selected Natura 2000 sites.
- Management schemes and methods for managing issues with private land in Natura 2000 network.
- Operational tools and systems for improving governance and management of Natura 2000 network.
- Development and establishment of a permanent, holistic monitoring system for habitats and species in all Natura 2000 sites to improve the consistency of monitoring under Article 17.
- Development and management of a Natura 2000 funding coordination unit.
- Development of a Habitat guide and a Red Data Book for Habitats of Cyprus.
- Development of the Red List for the Birds of Cyprus (https://pandoteira.cy/).

6.2.2 Projects funded by the Research Promotion Foundation (RPF)

6.2.2.1 BIODIRECTIVE

The project entitled 'Conservation Biology of 4 Priority Plant Species of Directive 92/43/EEC' (ENIΣX/0506) was implemented within the framework of Action 'Enhance' of the PENEK program of the Research Promotion Foundation (RPF). The total project budget was 115,502 Cyprus pounds, of which 57,538 pounds (50% of its total eligible budget) were co-funded by RPF. The project aimed at implementing high-level scientific research and specific in-depth study of the biology of four endangered endemic plant species of the Cyprus' flora: **Arabis kennedyae*, **Pinguicula crystallinα*, **Scilla lochiae* and **Scilla morrisii*. Specifically, the main objectives of the project were: a) the systematic monitoring of four priority Annex I (Directive 92/43/EEC) plant species, b) the genetic analysis of the population of **Arabis kennedyae*, c) the study of the vegetation growth of **Scilla morrisii*, d) the investigation of the poor fruit setting of **Scilla lochiae* and e) the study of nutrition physiology in **Pinguicula crystallina*.

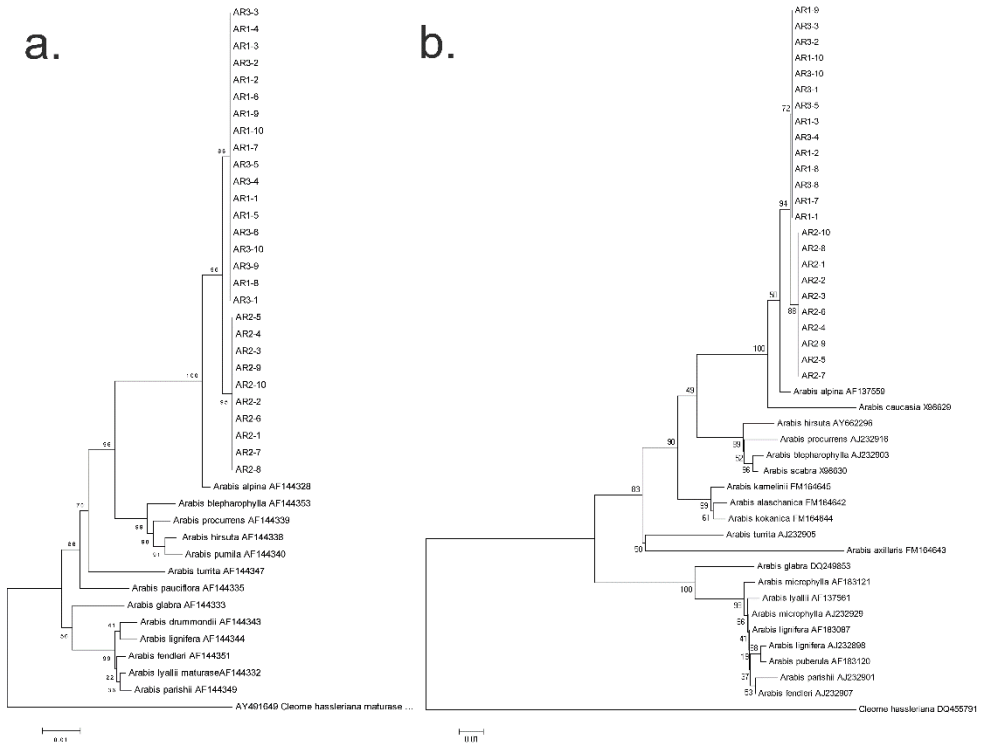

Fig. 6.4 Neighbor-joining phylograms showing the phylogenetic relation between *A. kennedyae* halpotypes. The numbers at each node represent bootstrap proportions. The trees represent the analysis of the matK (a) and ITS (b) sequences. (Source: Andreou et al. 2011).

The project was implemented by the Frederick Institute of Technology (Coordinating Beneficiary), the National and Kapodistrian University of Athens, the Department of Forests and the Environment Service, during December 2006 and December 2009.The main achievements of the project were:

- Many of the gaps concerning the biology of four of the most rare and endangered species of Cyprus were filled, which were used as 'tools' for managing and enhancing existing populations by the Department of Forests and the Environment Service (End Users).
- The Department of Environment and the Department of Forests had a unique, pilot, work study and research on the biology of four plant species, that were included in the Red Data Book of the Flora of Cyprus, and which were the basis for similar studies of other endangered species.
- Preparation and implementation for the first time of Plant Species Monitoring Plans in Cyprus.
- Carrying out, for the first time, genetic analysis on priority plant species of Cyprus (Fig. 6.4).
- Carrying out, for the first time, a Population Viability Analysis in plant species of Cyprus (Fig. 6.5) (Andreou 2012, Andreou et al. 2011, Andreou et al. 2015).

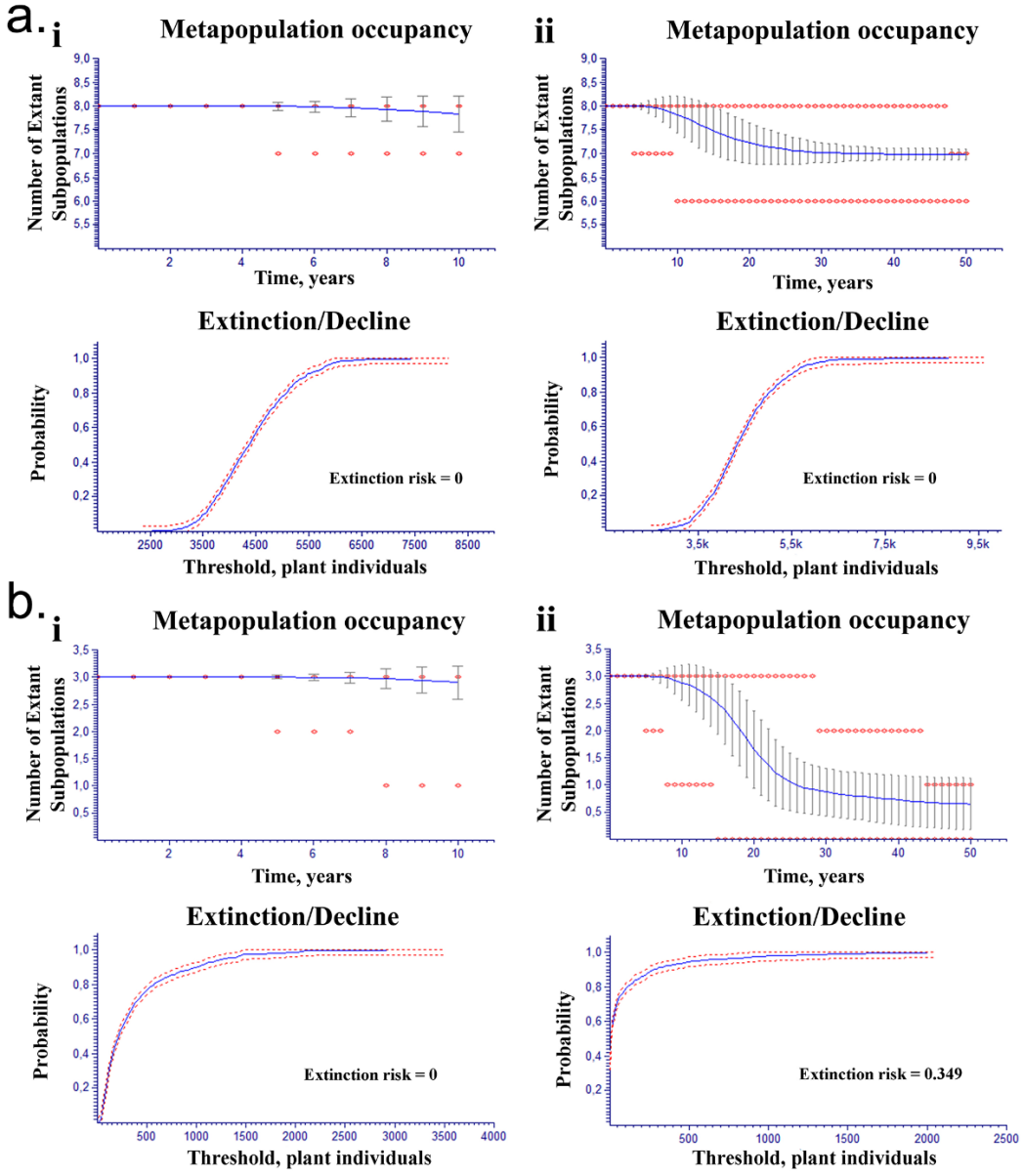

Fig. 6.5 a. Population Viability Analysis of *Scilla lochiae* for the next 10 (i) and 50 years (ii). b. Population Viability Analysis of *Scilla moriisii* for the next 10 (i) and 50 years (ii). The average (line), ±1 standard deviation and minimum and maximum (dots) number of extant populations are shown (Source: Andreou et al. 2015).

6.2.2.2 EX SITU

The project entitled '*Ex situ* conservation of local endemic species of occupied Cyprus' (ERYDI/0506/01) was implemented with funding from the Research Promotion Foundation (renamed to Research and Innovation Foundation). The total project budget amounted to 258,075 euro and was carried out from December 2006 to January 2011. The project carried out *ex situ* conservation activities to secure the survival and perpetuation of threatened local endemic plant species of Pentadactylos mountain range. The project was implemented by the Nature Conservation Unit of Frederick University (Coordinator), the Department of Forests and the Agricultural Research Institute (Fig. 6.6).

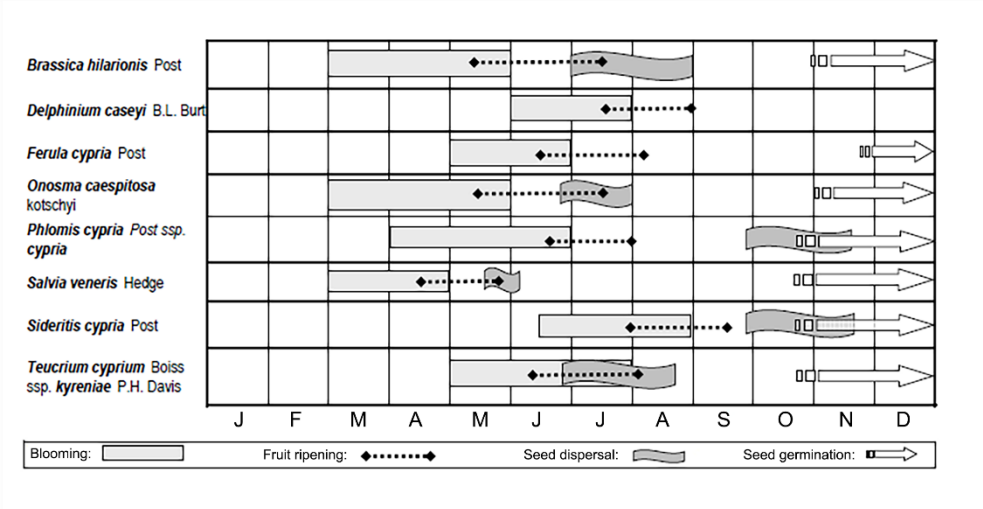

Fig. 6.6 Phenology of selected species of Pentadactylos mountain (Source: Kadis and Kounnamas 2011).

The most significant achievements of the project were:

- Establishment of a laboratory (NCU) for conducting research experiments on plant species.
- Identification of the studied plants in the field and collection of data on their habitat characteristics, prevailing environmental parameters, and potential threats.
- Study of plant phenology and collection of data on their reproductive strategy.
- Collection of seeds according to internationally accepted practices from seven studied species.
- Laboratory testing of seed germination, including investigation of temperature dependence, the presence of dormancy, and conditions for dormancy release by various promotive factors. The results of germination experiments were utilized in interpreting the reproductive strategy of the studied species and in the *ex-situ* conservation of the studied plants.
- Storage of seeds in the National Seed Bank of the Agricultural Research Institute.

- Provision of young plants to the Department of Forests for the establishment of the studied plants in living collections at the Department's Botanical Gardens (Kadis and Kounnamas 2011).

6.2.2.3 PAFOS FOREST

The project entitled 'Using mathematical modelling to examine the impacts of social and biological factors on the biodiversity of the Paphos Forest' (PENEK/SUPPORT/0308/42) was implemented within the framework of the National Framework Programme for Research, Technological Development and Innovation 2008 of the Research Promotion Foundation (RPF). The total budget of the project was 92,107 euro, of which 89,944 euro (98% of the total eligible budget) was funded by the RPF.

The project aimed at developing models to study the biological and social factors affecting the avifauna in the Paphos Forest. The project was implemented by the Nature Conservation Unit of Frederick Research Center (Coordinating Beneficiary), the Imperial College London of the United Kingdom, the Department of Forests, and the BirdLife Cyprus, during the period from August 2009 to October 2013. Within the framework of the project, models were also developed to assess the impacts of various factors on the avifauna of the Natura 2000 sites in Cyprus.

The most significant achievements of the project were:

- Collection of data from existing sources on various important social and biological factors within the Natura 2000 network, such as road network density and diversity of habitats.
- Quantification of the impacts of these factors on the avifauna of the Natura 2000 sites, specifically the number of species, using structural equation models based on possible correlations collected by consulting biodiversity experts in Cyprus.
- Understanding and highlighting the most important factors influencing bird diversity in these areas, with specific reference to the negative correlation between the number of bird species and network density, as well as the positive correlation between habitat diversity and area size with the number of bird species.
- Understanding and highlighting the most important factors influencing bird diversity within Pafos forest, particularly the negative correlation between road network density and the number of species (Mammides et al. 2015, Mammides et al. 2016).

6.2.2.4 TROODOS

The project entitled 'Impacts of Climate Change on the Local Endemic Plants of Troodos National Forest Park' (AEIFORIA/FYSI/0308(BE)/07) had a total project

budget of 109,720 euros, and it was implemented from September 2009 to October 2012. The project was implemented by the Nature Conservation Unit (through the Frederick Research Center – Coordinating Beneficiary), the Department of Forests and the National and Kapodistrian University of Athens. The project aimed to investigate the impacts of climate change on critical parameters of the reproductive biology of local endemic plant species in the Troodos National Forest Park.

Within this work, the following achievements were made:

- Data collection regarding the geographical distribution and biology of the studied species.
- Study of the geographical distribution of the populations of the studied plants through the recording of their locations (Fig. 6.7) and comparison of the data with other literature references.
- Determination of the Reproductive Potential and Relative Reproductive Success in most of the studied species, at different altitudinal sites. Seed collection from these sites was also conducted.
- Study of the seed germination behavior (collected from different altitudes and habitats) under a range of different temperature conditions.
- Identification of the most probable dispersal mechanism of the studied plants and determination of the possibility/ ease of colonization of higher altitude sites.
- Utilization of data from initial experimental growth control conditions and projected temperatures for 2080 to implement a complex seed germination control experiment of the studied plant taxa.
- Analysis of the above data and creation of a risk assessment map for the studied plants using the ArcGIS program. The map marked the areas of plant distribution and their potential future distribution.
- Formulation of proposals regarding the conservation of the studied plants and the management of the areas hosting them, taking into account the above analysis (Kadis and Kounnamas 2012).

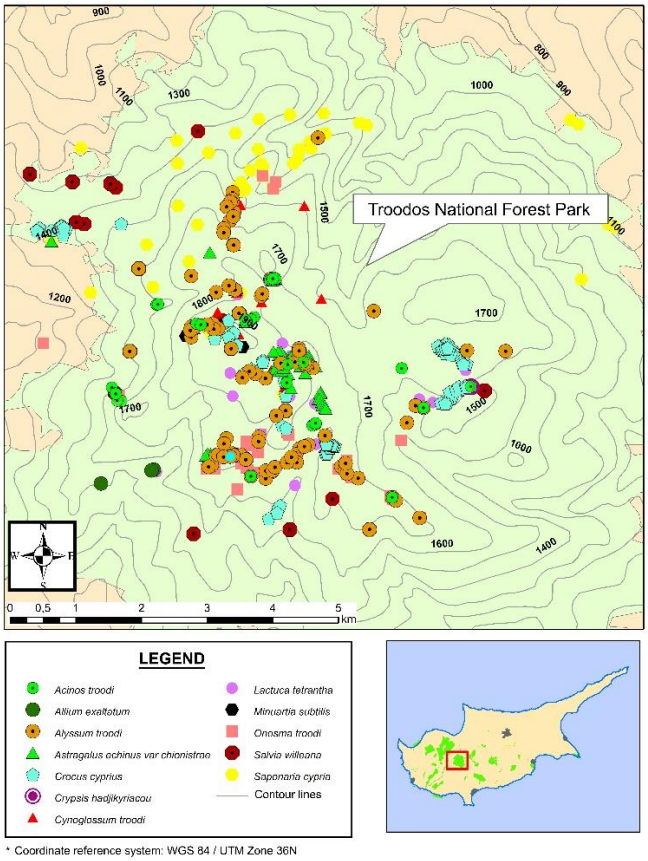

Fig. 6.7 Distribution map of selected plant species within the Troodos National Forest Park (Source: Kadis and Kounnamas 2012).

6.2.2.5 INVERTEBRATES

The project entitled 'Biodiversity patterns (flora and invertebrate fauna) regarding local climate change in Cyprus' (PENEK/0609/34) was implemented under the framework of program PENEK and funded by the Research Promotion Foundation. The total project budget was 111,230 euros. The general aim of the project was the ecological study of the flora, the Orthoptera and the hemerobious Lepidoptera and the effect of altitude gradient (local climate change) on their distribution, in two mountainous areas of Cyprus which are included in the Natura 2000 network (Troodos National Forest Park and Madari area). The project's partners were the Frederick Research Centre (Coordinating Beneficiary), the Ioannina University, the Exeter University, the Department of Forests and the Department of Environment. Its duration was 2010 – 2013.

The main achievements of the project were:

- Sampling of butterflies and 11 environmental factors in 60 randomly selected sites across four 500 m elevation zones, representing seven habitat types.
- Proofing that the number of flower heads was the most important factor favouring butterfly species richness and abundance and endemic butterfly richness, while soil humidity had a positive effect on species richness and abundance.
- The project showed that the mosaic vegetation (abandoned or non-agricultural crops with a strong presence of plant hedges and with natural vegetation mainly with low shrubs) is the richest in number of species and has a great ecological value. The riverside ecosystems are refuges especially for butterflies during summer season. Butterfly species richness has been correlated with the presence of water.
- The main environmental factors that determine the diversity patterns of diurnal Lepidoptera in the protected area of Madari are the altitude and the structure of the vegetation (trees), while in the Troodos National Forest Park it is the rocky substrate and the light intensity (Tzirkalli et al. 2019).

6.2.2.6 CEDRUS

The project entitled 'Vulnerability of the narrow endemic *Cedrus brevifolia* from Cyprus: Detection of genes and phenotypic diversity of traits linked to adaptation' (DIDAKTOR/0609/13) was implemented under the 'Research Framework Program, Technological Development and Innovation 2009-2010' (Priority Axis II: Human Resources Development – DOCTOR Program) of the Cyprus Research Promotion Foundation. The total project budget was 149,657 euro. The project focused on investigating the adaptation and adaptability of *C. brevifolia* through the correlation of various datasets: genetic diversity, morphological/anatomical characteristics, and ecological indicators (soil conditions and climatic measurements. The project was implemented by the Frederick Research Center (Coordinating Beneficiary), the French National Institute for Agricultural Research (INRA – Avignon), the Democritus University of Thrace and the Department of Forests, during August 2011 – January 2015.

The main achievements of the project were:

- Identification of 13 adaptive polymorphic genes, with a high polymorphism index (based on amino acids – SNPs), six of which were applied to study the genetic polymorphism and genetic structures of subpopulations of *C. brevifolia* within the natural boundaries of its distribution. At the same time, 28 morphological and 7 anatomical characteristics was measured from tree needles from each of the sampling plots (Eliades et al. 2014a).

- The genetic study of *C. brevifolia* which showed that despite the species' limited range and the fragmentation of its population into partial sets, it maintains satisfactory levels of diversity (Eliades et al. 2014a).
- The genetic differentiation of the fragmented parts potentially reflects the different origins of the segments of the *C. brevifolia* population, as remnants of earlier populations that were driven to the high peaks of the Paphos Forest for their survival amidst the changing climate and environmental conditions in the Mediterranean over the centuries. Furthermore, the theory of local adaptive mechanisms of species individuals to irregular local conditions should not be dismissed (Eliades et al. 2014a).
- The species' diversity in terms of morphological and anatomical characteristics in needle traits reflects two simultaneous patterns: a geographic trend and a trend based on the local adaptation of trees in different sampling plots (Eliades et al. 2014b).

6.2.2.7 EVOIKO

The project entitled "Vulnerable Coastal Habitats of Cyprus" (PENEK/16/2002) was implemented under the framework of the Cyprus Young Researchers Support Program (PENEK). The project focused on the identification, recording, description, and ecological evaluation of 11 vulnerable coastal dune and halophytic habitats in Cyprus. It also included the development of a pilot management plan in the form of management proposals and aimed to increase scientific knowledge about the vegetation of the target habitat types. The project was implemented by the Agricultural Research Institute (Coordinating Beneficiary), the National and Kapodistrian University of Athens (Department of Biology, NKUA), the University of Ioannina (Department of Environmental and Natural Resources Management), the Department of Environment of the Ministry of Agriculture, Rural Development and Environment, and the Pan-Cyprian Environmental Movement “Enarmonisis”, during the period of January 2003 – December 2005.

This project formed the basis for the preparation of a PhD dissertation titled "Conservation Biology of Threatened Coastal Habitats: Flora, Vegetation, Ecology, and Management" (2005), completed at the Department of Biology, National and Kapodistrian University of Athens. It also laid the groundwork for the OIKOAKT project (2006-2007), during which the book "Ecology of Threatened Coastal Ecosystems of Cyprus" (Hadjichambis and Della 2007) was published by the Research Program Results Publications of the Research Promotion Foundation.

The main achievements of the project were:

- Identification of the areas in Cyprus where the 11 target habitat types are found.
- Expansion of knowledge on the flora of the target dune and halophytic ecosystems, including the study of life forms and their distribution, as well as the geographic spread of species across the botanical divisions of Cyprus. Many were new reports for dunes or new records for Cyprus's botanical divisions (Hadjichambis et al. 2004).
- Discovery and phytosociological-phytoecological description of 50 plant communities in dune ecosystems and 33 in halophytic ecosystems, many of which were reported for the first time in Cyprus, broadening knowledge about their distribution in Southeastern Europe. Each plant community was described in terms of floristic structure, topography, and soil data, including their distribution in Cyprus, conservation status, synanthropism, and observed human pressures.
- The ecological conditions of the identified plant communities were another crucial contribution, marking the first such research in Cyprus. The study of soil factor differentiation within dune ecosystems significantly enhanced scientific understanding of their ecology.
- Increased knowledge of the ecology and biology of species recorded in the target ecosystems.
- The recording and assessment of human-induced pressures and threats at the level of plant samplings, plant communities, and regions contributed to a greater scientific understanding of the ecology of human impacts and threats on these ecosystems.
- The scientific data uncovered provided a critical mass of knowledge that formed the basis for a series of actions, such as monitoring of species, habitats, and ecosystems, elaboration of management plans, defining of nature protection zones, restoration of species, habitats, and ecosystems, and implementation of environmental education, awareness, and information programs, as well as plans for the utilization of plant genetic resources.
- Importantly, this research introduced several innovative methodological approaches that can be applied in future studies by other researchers of coastal ecosystems or habitats and ecosystems in general. These innovations include:
  - The integration of phytosociological-phytoecological methodology with data on human pressures and threats, resulting in the creation of frequency and intensity tables for each described plant community.
  - The semi-quantitative recording and assessment of human pressures and threats.
  - The sequences of life forms combined with biomes improved the understanding of life form differentiation, especially in studies concerning plant communities of a specific habitat type or plant communities with similar ecological requirements.

- Classification of species into synanthropic categories and the calculation of community synanthropism provided a pioneering approach to human impact on plant community level.
- The Relative Classification Index (RCI) of soil characteristics allowed a comparative examination of soil results in a unified classification system, enabling the creation of charts for each studied plant community.
- The proposal of the Impact Value (IV), Total Impact Value (TIV), and Community Impact Value (CIV) which represented a significant contribution to the evaluation of pressures on habitats (Hadjichambis 2005).

6.2.3 Other funding sources' projects

6.2.3.1 ENSCONET

The project entitled 'European Native Seed Conservation Network' was implemented within the framework of the 6th Framework Programme of the EU, during the period 2004 – 2009. The objective of the project was to improve quality, coordination and integration of European seed conservation practice, policy and research for native plant species and to assist EU conservation policy and its obligations to the Convention on Biological Diversity and its Global Strategy for Plant Conservation. The project involved the following partners: Royal Botanic Gardens, Kew (Coordinator Beneficiary), National and Kapodistrian University of Athens, Institute of Botany, Slovak Academy of Sciences, Bratislava, Budapest Zoo & Botanical Garden, Mediterranean Agronomic Institute of Chania, IMGEMA- Jardin Botanico de Cordoba, Botanical Garden, Trinity College Dublin, Jardin Botanico Gran Canaria, Agricultural Research Institute, Cyprus, Universidad Politecnica de Madrid, National Botanic Garden Belgium Meise, Museun National d'Histoire Naturelle, Paris, University di Pavia/Centro Flora Autoctona della Lombardia, University di Pisa, Orto Botanico, Jardim Botanico de Soller, Museo Tridentino di Scienze Naturali Trento, Jardin Botanic, Universitat de Valencia, Universitat Wien, Botanical Garden Polish Academy of Sciences Warsaw, Botanischer garten und Botanisches Museum Berlin-Dahlem, FU Berlin, University of Helsinki, Jardim Botanico - Fundatio da Universidade de Lisboa, Natural History Museum, University of Oslo, Institute of Botany - Bulgarian Academy of Sciences, University of Natural Resources and Applied Life Sciences Vienna, Musue National d'Histoire Naturelle Luxembourg, Conservatoire et Jardin Botaniques Geneve, RIBES- Rete Italiana Banche del Germoplasma per le Pianti Spontanee Minacciate, and NCU of Frederick University.

The main achievements of the project were as follows:

- Fostered a spirit of collaboration among European Seed Banks with indigenous species.
- Established a unified approach for conserving seeds of indigenous species in seed banks across Europe.
- Created a platform for discussion and exchange of experiences.
- Facilitated interaction between activities and organizations for better effectiveness and reducing overlap of efforts.
- Appointed a lead partner in the European Strategy for Plant Conservation (ESPC).
- Conducted an analysis of collections and facilities of indigenous Seed Banks in Europe.
- Prepared and published online two handbooks for collection and curation of indigenous species seeds in Seed Banks (ENSCONET 2009a, ENSCONET 2009b).
- Developed an online educational tool for Seed Banks (Mueller 2009).

6.2.3.2 TREE-SEEDS

The project titled ‘Global Tree Seed Bank Project’ (GW-GTSBP-Europe-FU) was implemented with funding from the Global Tree Seed Bank Project. The total budget of the project amounted to 381,235 euro and was implemented from July 2015 to March 2018. The project was carried out by the Royal Botanical Gardens Kew (Coordinating Beneficiary), with the lead partner in Cyprus being the Nature Conservation Unit of Frederick University, and collaborating entities included the Department of Forests and the Agricultural Research Institute. The project was a global initiative aimed at ensuring the survival of over 2000 tree species, including the rarest, most threatened, and most valuable trees in the world. The goal of the project was to collect and conserve in seed banks at least 200 indigenous tree species of Europe (https://www.kew.org/science/our-science/projects/global-tree-seed-programme).

The most important achievement of the project for Cyprus was the collection of seeds (2 seedlots) from 40 native trees or native shrubs, within 3 years. The collection was carried out according to the "Seed Collecting Manual for Wild Species" of the European Native Seed Conservation Network (ENSCONET).

6.3 Conclusions

The summary reference to the achievements of the aforementioned projects highlights the volume of efforts and the substantial steps taken over the last 25 years in relation to biodiversity conservation and operation of the Natura 2000 Network in Cyprus.

The majority of the projects focused on the implementation of significant conservation measures, both *in situ* and *ex situ*, with the exception of one (iLIFE-TROODOS), which exclusively concerned a campaign to raise awareness about the natural values of a specific site of the Network and the ecosystem services it provides to humans. However, all the projects, alongside conservation actions, also included activities aimed at informing and raising public awareness, or, more specifically, environmental education and training within the framework of educational programs.

The main results of the projects presented in this work are summarized in Table 6.2. Overall, within the framework of the completed projects:

- A total of 38 sites were proposed and incorporated into the Natura 2000 Network as Sites of Community Importance (both terrestrial and marine) and 8 sites as Special Protection Areas, following relevant documentation.
- 47 habitat types were mapped, of which five are new additions to Annex I of the Habitats Directive.
- 109 bird species were recorded, of which 6 are new additions to Annex I of the Birds Directive.
- Nine mammals were recorded (two of which are new additions), 5 reptiles (three of which are new additions), 1 fish, and 2 invertebrates (one of which is a new addition) listed in Annex II of the Habitats Directive.
- Ten plant species were added to Annex II of the Habitats Directive, 8 of which are classified as priority species.
- Management measures were implemented for 15 habitat types and 60 species (*in situ*) in the majority of sites of the Natura 2000 Network.
- 14 Monitoring Plans for habitat types and 8 Monitoring Plans for flora and fauna species were developed.
- Seven Action Plans for habitat types and 11 Action Plans for flora and fauna species were elaborated.
- Five Management Plans for habitat types and three Management Plans for plant species within Natura 2000 areas were created.
- A significant number of seed samples were stored in seed banks as part of *ex situ* conservation for 14 plant species and the characteristic species of five habitat types.

The implementation of targeted conservation measures within the framework of the projects carried out has significantly contributed either to improving/ filling knowledge gaps that existed regarding specific species and habitat types, or to improving their conservation status, with the ultimate goal of ensuring their survival and perpetuation in the future. Furthermore, these actions are mandated by the Habitats and Birds Directives within the context of the proper operation of the Natura 2000 Network. In other words, the implementation of the projects also fulfilled the obligations of the Republic of Cyprus arising from EU law.

Table 6.2 Aggregate results of projects implemented by habitat type and by species. Where: P=plant, B=bird, I=invertebrate, R=reptile, M=mammal (Part 1).

Species or H.T. code	Name of species or Habitat Type (H.T.)	Species Type	Study of Biology	Structure Study	Genetic Analysis	Management Plan	Monitoring Plan	Action Plan	Monitoring	*In situ* measures	*Ex situ* measures	Restoration
1120	*Posidonia* beds (*Posidonion oceanicae*)			+			+		+	+		
1210	Annual vegetation of drift lines								+	+		+
1310	*Salicornia* and other annuals colonizing mud and sand			+			+	+		+		+
1410	Mediterranean salt meadows (*Juncetalia maritimi*)			+			+			+		+
1420	Mediterranean and thermo-Atlantic halophilous scrubs (*Sarcocornetea fruticosi*)						+			+		+
2110	Embryonic shifting dunes			+					+	+		+
2230	Malcolmietalia dune grasslands			+					+	+		+
2240	Brachypodietalia dune grasslands with annuals			+					+			
1520	Iberian gypsum vegetation (*Gypsophiletalia*)			+		+	+		+	+	+	
3170	Mediterranean temporary ponds			+			+		+	+		
5220	Arboresent matorral with *Ziziphus*			+		+	+	+	+	+	+	+
62B0	Serpentinophilous grassland of Cyprus			+			+		+	+		
6420	Mediterranean tall humid grasslands of the Molinio-Holoschoenion			+				+				
6460	Peat grasslands of Troodos								+			
92A0	*Salix alba* and *Populus alba* galleries			+				+	+			
92C0	*Platanus orientalis* and *Liquidambar orientalis* woods (*Platanion orientalis*)			+			+	+	+	+		+
92D0	Southern riparian galleries and thickets (Nerio-Tamaricetea and Securinegion tinctoriae)			+				+				
93A0	Woodlands with *Quercus infectoria* (Anagyro foetidae-Quercetum infectoriae)						+		+			+
9390	Scrub and low forest vegetation with *Quercus alnifolia*			+		+	+		+	+		
9530	(Sub-) Mediterranean pine forests with endemic black pines			+		+	+		+	+	+	
9560	Endemic forests with *Juniperus* spp.			+		+	+		+	+	+	
9590	*Cedrus brevifolia* forests (*Cedroseum brevifoliae*)			+	+	+	+	+	+	+	+	+
2103	*Arabis kennedyae*	P	+		+		+		+	+	+	+
2131	*Astragalus macrocarpus* subsp. *Lefkarensis*	P	+		+	+	+	+	+	+	+	
2106	*Brassica hilarionis*	P									+	
2250	*Centaurea akamantis*	P	+		+	+	+		+	+	+	
4082	*Crepis pusilla*	P						+	+	+	+	
2306	*Crocus cyprius*	P	+						+		+	
-	*Delphinium fissum* subsp. *caseyi*	P									+	
2329	*Ophrys kotschyi*	P	+		+	+	+	+	+	+	+	
2209	*Phlomis brevibracteata*	P						+	+	+	+	
2210	*Phlomis cypria*	P									+	
2227	*Pinguicula crystallina*	P	+				+		+	+		
4099	*Salvia veneris*	P									+	
2213	*Sideritis cypria*	P									+	
2283	*Scilla lochiae*	P	+				+		+	+	+	+
2296	*Scilla morrisii*	P	+				+		+	+	+	

Table 6.2 Aggregate results of projects implemented by habitat type and by species. Where: P=plant, B=bird, I=invertebrate, R=reptile, M=mammal (Part 2).

Species or H.T. code	Name of species or Habitat Type (H.T.)	Species Type	Study of Biology	Structure Study	Genetic Analysis	Management Plan	Monitoring Plan	Action Plan	Monitoring	*In situ* measures	*Ex situ* measures	Restoration
-	*Hierophis cypriensis*	R	+				+	+	+			
4407	*Natrix natrix cypriaca*	R	+					+	+	+		
1078	*Callimorpha quadripunctaria*	I	+					+	+	+		
4023	*Propomacrus cypriacus*	I	+					+	+	+		
1310	*Miniopterus schreibersii*	M							+	+		
1307	*Myotis blythii*	M							+	+		
1321	*Myotis emarginatus*	M							+	+		
1306	*Rhinolophus blasii*	M							+	+		
1305	*Rhinolophus euryale*	M							+	+		
1304	*Rhinolophus ferrumequinum*	M							+	+		
1303	*Rhinolophus hipposideros*	M							+	+		
4002	*Rousettus aegyptiacus*	M						+	+	+		
1224	*Caretta caretta*	R	+					+				
1227	*Chelonia mydas*	R	+					+				
A229	*Alcedo atthis*	B							+	+		
A404	*Aquila heliaca*	B							+	+		
A060	*Aythya nyroca*	B							+	+		
A396	*Branta ruficollis*	B							+	+		
A133	*Burhinus oedicnemus*	B							+	+		
A403	*Buteo rufinus*	B							+	+		
A224	*Caprimulgus europaeus*	B							+	+		
A243	*Calandrella brachydactyla*	B							+	+		
A469	*Certhia brachydactyla dorotheae*	B							+	+		
A138	*Charadrius alexandrinus*	B	+						+	+		
A081	*Circus aeruginosus*	B							+	+		
A082	*Circus cyaneus*	B							+	+		
A083	*Circus macrourus*	B							+	+		
A231	*Coracias garrulus*	B							+	+		
A773	*Egretta alba*	B							+	+		
A026	*Egretta garzetta*	B							+	+		
A447	*Emberiza caesia*	B							+	+		
A100	*Falco eleonorae*	B							+	+		
A103	*Falco peregrinus*	B							+	+		
A189	*Gelochelidon nilotica*	B							+	+		
A078	*Gyps fulvus*	B							+	+		
A707	*Hieraaetus fasciatus*	B							+	+		
A131	*Himantopus himantopus*	B	+						+	+		
A022	*Ixobrychus minutus*	B							+	+		
A338	*Lanius collurio*	B							+	+		
A339	*Lanius minor*	B							+	+		
A433	*Lanius nubicus*	B							+	+		
A242	*Melanocorypha calandra*	B							+	+		
A023	*Nycticorax nycticorax*	B							+	+		
A467	*Oenanthe cypriaca*	B							+	+		
-	*Parus ater cypriotes*	B							+	+		
A072	*Pernis apivorus*	B							+	+		

These efforts were recognised by the EU funding mechanisms, resulting in the projects PLANT-NET CY, JUNIPERCY, and LIFE-KEDROS standing out and being awarded, competing against a multitude of other European projects. Additionally, the projects LIFE-RIZOELIA, PLANT-NET CY, JUNIPERCY, and COMANACY have been included in the EU publication "Bringing nature back through LIFE" (Johnson et al. 2020). This publication highlights projects that played a crucial role in nature and biodiversity protection since the inception of the program in 1992. Furthermore, the project LIFE-KEDROS was included in the EU publication "Ready, steady, green!: LIFE helps farming and forestry adapt to climate change" (Toland et al. 2019), which showcases the EU's contribution to the adaptation of agriculture and forestry to climate change. It is also mentioned in the article from the European Climate, Infrastructure and Environment Executive Agency regarding the adoption of the Nature Restoration Law (https://cinea.ec.europa.eu/news-events/news/new-nature-restoration-law-boosts-biodiversity-and-climate-action-across-europe-2023-07-12_en).

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Chapter 7
Genetics of Inherited Endocrine Disorders in Cyprus: The present and the future

Vassos Neocleous, Pavlos Fanis and Leonidas A. Phylactou

Cite this chapter:

Neocleous, V., Fanis, P. and Phylactou, L. A. (2025). Genetics of Inherited Endocrine Disorders in Cyprus: The present and the future. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 185-206). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 7
Genetics of Inherited Endocrine Disorders in Cyprus: The present and the future

Vassos Neocleous, Pavlos Fanis and Leonidas A. Phylactou

Molecular Genetics, Function & Therapy Department, The Cyprus Institute of Neurology & Genetics 6 Iroon Avenue, 2371 Ayios Dometios, Nicosia, Cyprus

Keywords: Genetics, endocrine disorders, Cyprus

Chapter Abstract: All globally reported inherited diseases include a genetic connection with variable pathogenic status. This chapter provides information of current available data and future endeavors in Cyprus regarding inherited endocrine disorders. To date, inherited rare endocrine disorders that have been reported include Congenital Adrenal Hyperplasia (CAH), Multiple Endocrine Neoplasia (MEN) type 2, Disorders of Sexual Differentiation (DSD), Glucose and Insulin Homeostasis and Premature and Delayed puberty. During the last 20 years for most of the of the above-mentioned disorders, there has been extensive investigations via epidemiological and genetic studies. Currently, in Cyprus several ongoing research programs look for the involvement of genomic, epigenetic and molecular factors, as well as the discovery of potential biomarkers for prognosis and diagnosis. Advances in understanding the genetic mechanisms behind these clinical entities will aid towards the development of diagnostic tests, innovative therapies and personalized medicine to prevent disease onset. Even though it is impossible to envisage fully the future, it is possible to predict that cutting-edge technologies hold a great future promise for medicine and healthcare. It is certain that innovative approaches will be applied in the clinical practice in Cyprus health care system with certain benefits for patients with rare endocrine disorders.

7.1 Introduction

The current advancements in biotechnology and genetics revolutionized the approach towards inherited and chronic diseases, including endocrine disorders with a genetic basis. These breakthroughs have empowered the early diagnosis of conditions, prediction of potential risks and development of more targeted treatment strategies [1]. For over two decades, Sanger sequencing ran as the gold standard in genetic testing. It played a pivotal role in deciphering the molecular etiologies of countless monogenic and inherited diseases. However, the landscape of genetic testing is undergoing a transformative shift with the emergence of next-generation sequencing (NGS) technology. This powerful tool offers a high-throughput approach, sequencing millions of DNA fragments simultaneously, compared to Sanger's targeted analysis. Beyond any doubt, the arrival of NGS has been a game-changer in genomics research and diagnostics. Unlike Sanger sequencing, which analyzes one fragment at a time, NGS is incredibly fast and accurate, empowering researchers to analyze entire genomes or target specific regions with remarkable efficiency. NGS can uncover rare genetic variants that were previously undetectable, leading to the diagnosis of entirely new clinical entities – diseases with previously unknown genetic causes. This explosion of knowledge has deeply revolutionized the field, significantly contributing to our cumulative understanding of genetics. The impact of NGS extends far beyond research and as above stated, has a profound effect on patient care. By enabling the identification of the underlying genetic causes of disease, NGS allows physicians to provide more accurate diagnoses and personalized treatment plans. This not only benefits affected patients but also their families, who can receive valuable information about their own genetic risks.

To date, genome the use of sequencing by NGS for the diagnosis of inherited Endocrine Disorders in Cyprus is the emerging method of choice for the identification of the biological mechanisms and malfunctions of particular genes. Consequently, genetic testing can be especially helpful particularly when the standard hormone tests produce unclear results with unanswered questions. Unlike hormone tests, which give a snapshot of the current hormone levels they provide insights into genetic factors that recognize inheritable mechanisms. Treatment plans based on premature diagnosis for common health issues such as thyroid disease and sporadic adrenal tumors can significantly benefit an individual’s health. Numerous molecular testing choices are accessible to aid in diagnostic validation and risk assessment for family members.

Since January 2020, the accredited with ISO15189 Department of Molecular Genetics, Function and Therapy (MGFT) at the Cyprus Institute of Neurology and Genetics (CING) has become an affiliated reference center (RC) for the European Reference Network on Rare Endocrine Conditions (Endo-ERN) (https://endo-ern.eu/reference-centre/department-of-molecular-genetics-function-and-therapy-the-cyprus-institute-of-neurology-and-genetics/). This designation allows MGFT to

collaborate with other experts in the Endo-ERN network to share knowledge, improve patient care and develop new treatments for these complex diseases. At present, MGFT is participating in three of the Main Thematic Groups of the Endo-ERN. These groups focus on adrenal gland disorders, hypothalamic and pituitary condition, conditions affecting the hypothalamus and pituitary gland, sex development and maturation, disorders of sex development and maturation. By participating in these groups, MGFT is contributing to the Endo-ERN's overall objective of improving access to high-quality healthcare for patients with rare hormonal disorders. On this matter, MGFT along with the other Reference Centers (RCs) across Europe actively contributes to the continuous education programs that are frequently offered virtually or organized in the setup of scientific meetings by the Network. Moreover, MGFT is in direct contact with a several other RCs and frequently shares and receives valued clinical and scientific data so as to certify that the premium care is made obtainable to the patients both in the vicinity and across Europe. In addition, MGFT has also established basic science research program on the molecular mechanisms of pubertal development and epidemiological surveillances on specific rare endocrine disorders such as congenital adrenal hyperplasia (CAH) [2-5]. The involvement of MGFT on the diagnosis and research of inherited endocrine conditions dates back to the mid-2000s. Since then, the Department has been applying novel methodologies at its state-to-the-art facilities, allowing to provide comprehensive diagnoses for a wide range of inherited endocrinopathies. Such disorders include CAH [3, 6], disorders of sexual development [7-11], multiple endocrine neoplasia (MEN) type 2A and 2B [12], genetic conditions causing hypogonadotropic hypogonadism [13, 14], precocious and delayed puberty [4, 15-18], maturity onset diabetes of the young (MODY) [19], cases of obesity and various other less common disorders [20, 21]. The diagnosis of inherited rare conditions has always been a difficult challenge. Although, the traditional genetic testing methods provide crucial tools in the daily practice for both pediatric and adult endocrinology, they often have limitations. In many cases, the accuracy of assessing a patient’s phenotype and the effective management of the disease relies heavily on these tests. However, the general diagnostic outcome in the MGFT Department has undergone a significant transformation in recent years. The implementation of NGS assays has revolutionized the ability to identify the underlying genetic basis of complex endocrine disorders in Cypriot patients.

7.2 Endocrine Diagnostic Services at MGFT, CING: An ENDO-ERN Affiliated Reference Center

7.2.1 Diagnosis of Congenital Adrenal Hyperplasia (CAH) Resulting from Pathogenic Variants in the CYP21A2 Gene

In January 2006, the MGFT Department at CING first introduced a diagnostic service for the *CYP21A2* gene in Cyprus. This analysis aimed to diagnose of 21-hydroxylase deficiency (21-OHD), the predominant cause of CAH [22].

The spectrum of phenotypes associated with CAH, resulting from mutations in the *CYP21A2* gene, includes a wide range of clinical manifestations. At one end of the spectrum are the rare severe forms, namely salt-wasting (SW) and simple virilizing (SV) CAH [23]. These forms often manifest with significant prenatal virilization in affected females, presenting substantial challenges in both diagnosis and management.

On the other end of the spectrum lies the non-classic form of CAH, which is both milder and more prevalent compared to its severe counterparts. Individuals with the non-classic form may experience less pronounced symptoms and usually do not exhibit the characteristic of salt-wasting or virilizing features seen in the classic forms. However, despite the milder nature of the non-classic form, it remains a significant clinical concern due to its potential impact on health and quality of life. This spectrum of clinical phenotypes underscores the complex nature of CAH and highlights the importance of comprehensive diagnostic approaches for accurate classification and appropriate management of affected individuals. Early detection and intervention are crucial in mitigating the long-term health implications associated with CAH, irrespective of the severity of the clinical presentation [24]. Both classic SW and SV forms of the disorder are rare and characterized by prenatal virilization in females, their projected frequency in most populations range between 1:10,000 to 1:20,000 [24]. Currently, the prevalence estimates for the non-classic and milder form of the disorder, characterized by the absence of glucocorticoid deficiency varies between 1 in 100 to 1 in 500 live births [3, 24]. Typically, females with the non-classic form of the disorder exhibit compound heterozygosity, wherein they carry two mild pathogenic variants in the *CYP21A2* gene. This presentation is often characterized by symptoms such as hyperandrogenemia, hirsutism, premature puberty in childhood and potential infertility in adulthood [25]. Numerous studies, including one conducted by the MGFT team, have calculated the carrier frequency of *CYP21A2* variants in the general population to range from 1:10 to 1:25 individuals [2]. Identifying heterozygous carriers of the *CYP21A2* gene can also be attained through assessing ACTH-stimulated 17-OH Progesterone (17-OHP) levels. However, it's important to note that, generally, this particular test exhibits a higher degree of overlap with values observed in the healthy population [26]. Recent studies, including studies conducted by the MGFT Department, has indicated that a notable proportion of female carriers of the *CYP21A2* gene may face an elevated risk

of developing hyperandrogenemia. Additionally, variations have been observed in hormonal levels among heterozygous carriers, non-carriers, and females with non-classic congenital hyperplasia [27-30]. To date, the MGFT Department has performed over 1,200 diagnostic analyses of the *CYP21A2* gene [31]. These analyses primarily originated from Cyprus' national reference hospitals, with some contribution from private healthcare providers. Most patients tested for *CYP21A2* mutations were females experiencing hyperandrogenism symptoms before or around puberty. These symptoms included early pubic hair development, advanced bone age, severe acne or excessive body hair (hirsutism), with or without irregular periods. Importantly, these females did not show signs of virilization (masculinization) but had elevated 17-OHP levels.

As depicted in Figure 7.1, this collaboration led to a number of publications. These publications primarily focused on clinical and genetic findings related to *CYP21A2* pathogenic variants [3, 28, 29, 32-40], epidemiological data concerning the occurrence of *CYP21A2* in the local population [2]. Additionally, further scientific insights regarding the multiallelic and tandem RCCX complex in the MHC class III region of chromosome 6p21.3 were documented [41, 42].

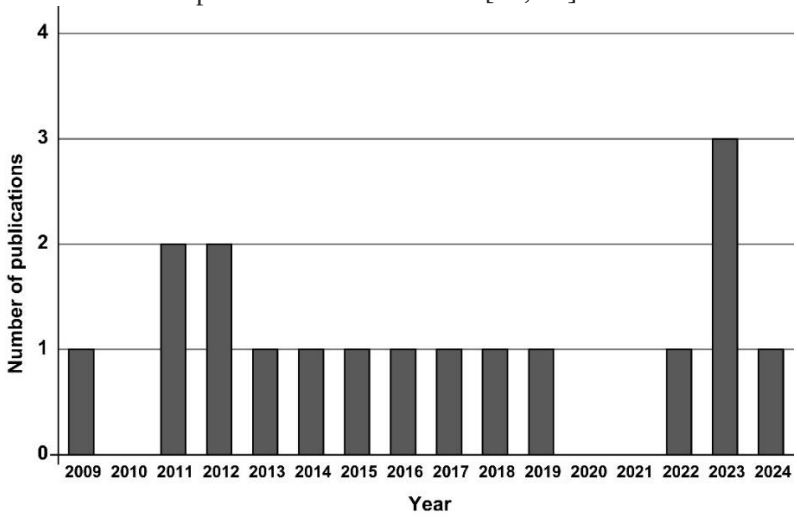

Fig. 7.1 Since 2009, the MGFT Department of the Cyprus Institute of Neurology and Genetics has published a total of sixteen papers covering comprehensive clinical, genetic, epidemiological and other scientific aspects of CAH in Cyprus.

As outlined in the recent CAH Best Practice Guidelines [23], the MGFT Department is presently employing the gold standard methods for genetic investigation of the *CYP21A2* gene. These methods include Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) [2, 3, 29, 41, 42].

Therefore, by combining Sanger sequencing, which detects exact nucleotide DNA changes, with MLPA, which identifies large gene deletions or duplications, doctors

gain a complete picture of the genetic variations that might cause 21-hydroxylase deficiency, leading to a more accurate diagnosis. In its ongoing commitment to enhancing genetic services, the MGFT Department has recently also implemented *TaqI* digestion for the detection of rare duplications and deletions in the *CYP21A2* gene [43]. This combined method offers superior sensitivity compared to traditional MLPA, not only excelling at rare copy number variations but also identifying specific gene deficiencies like chimeric *CYP21A1P/CYP21A2* and *TNXA/TNXB*. Consequently, this approach effectively addresses limitations inherent to Sanger sequencing and traditional MLPA techniques.

The MGFT Department recently conducted a comprehensive analysis of *CYP21A2* gene variations in Cypriot patients. This analysis is documented in the publication "Genetic diagnosis of endocrine disorders in Cyprus through the Cyprus Institute of Neurology and Genetics: an ENDO-ERN Reference Center [31]. A total of 571 pathogenic variants in the *CYP21A2* gene linked to CAH were identified with the key finding being that the p.Val281Leu variant is the most prevalent, present in over 62% of the studied patients (n=418). Consequently, a significant majority of *CYP21A2* variations causing CAH in the Cypriot population stems from this specific variant. Further details about the *CYP21A2* gene frequencies are provided in Figure 7.2. While CAH is an autosomal recessive disorder and one would not expect patients to exhibit clinical manifestations of the disease, some carriers, particularly females show clinical symptoms similar to those with two copies of the mutated *CYP21A2* gene [28, 29]. In addition to the milder CAH form, 18 patients of Cypriot descent were diagnosed with the severe classic type. Neocleous et al. from the MGFT Department have published detailed manuscripts exploring the link between a patient's genetic makeup (genotype) and the severity of congenital adrenal hyperplasia (CAH), specifically focusing on the most severe forms, salt-wasting (SW) and simple virilizing (SV) [3, 38]. There are numerous studies that define the exerted pathogenic effect(s) of the identified *CYP21A2* gene variants and align with the clinical signs and symptoms observed in patients identified by the MGFT Department [3, 33, 36-38]. The Department of MGFT is actively collaborating with the Endo-ERN network on this topic and regularly share results with other research centers through webinars, meetings and electronic databases like the European Registries for Rare Endocrine Conditions (EuRRECa) (https://endo-ern.eu/registries/eurreca/). The MGFT Department also contributed to a recent European study examining expert opinions on the significance of prenatal dexamethasone (Pdex) treatment for CAH caused by 21-hydroxylase deficiency [39]. For further information on the prevalence and effects of *CYP21A2* gene variants, one can refer to resources like the Human Cytochrome P450 Allele Nomenclature database (https://www.pharmvar.org/htdocs/archive/cyp21.htm) and the Human Gene Mutation Database (https://www.hgmd.cf.ac.uk/ac/all.php). Additionally, the European Molecular Genetics Quality Network (EMQN) has recently published best practice guidelines for molecular genetic testing and reporting of 21-hydroxylase deficiency, which offer valuable insights into this

area [23].

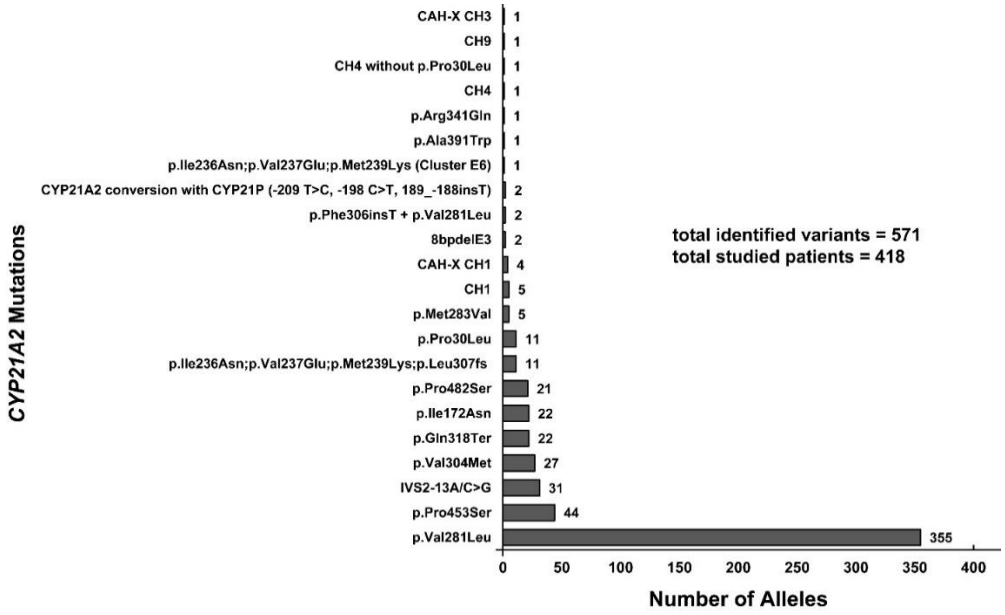

Fig. 7.2. Prevalence of the different *CYP21A2* variants in the Cypriot population. A total of 571 pathogenic variants were identified, with p.Val281Leu being the most frequent (62.17%) in the patients studied (n=418).

7.2.2 Diagnosis of Multiple Endocrine Neoplasia type 2 in Cyprus: The role of pathogenic defects in the RET proto-oncogene

Multiple endocrine neoplasia type 2 (MEN2) syndrome is a rare, inherited disorder characterized by the development of tumors in several hormone-producing glands such as the thyroid, parathyroid and adrenal glands [44]. It follows an autosomal dominant inheritance pattern that is mainly associated with cancer tumors in the thyroid gland, specifically medullary thyroid carcinoma (MTC), parathyroid tumors that lead to excessive parathyroid hormone production and hypercalcemia and pheochromocytoma as a result of overproduction of adrenaline in the adrenal glands [45, 46]. Patients with MEN2 can manifest one of the three discrete forms identified as MEN2A, MEN2B and Familial medullary thyroid carcinoma (FMTC). These forms depend on the severity and specific location of the mutations in the *RET* proto-oncogene which plays a critical role in cell growth and development. Pathogenic variants in the RET proto-oncogene disrupt its normal function, leading to the uncontrolled growth of cells and tumor formation in MEN2 [45, 47]. The American

Thyroid Association (ATA) and the European Thyroid Association (ETA) have classified known disease-causing mutations in the *RET* gene associated with MEN2 into three risk categories: highest (ATA-HST), high (ATA-H), and moderate (ATA-MOD). Interestingly, most of these mutations are located within specific regions of the *RET* gene, namely exons 10, 11, 13, 14, 15, and 16 [46, 48-50]. Since 2002, the MGFT Department has been a valuable resource for patients with suspected MEN2. Accredited with ISO 15189 for *RET* proto-oncogene testing, has performed over 500 tests to help diagnose individuals exhibiting symptoms like medullary thyroid carcinoma, pheochromocytoma, hyperparathyroidism, and cutaneous lichen amyloidosis. MGFT's ongoing research has identified *RET* proto-oncogene pathogenic variants associated with MEN2 in 58 out of 517 patients tested (11.2%). These patients come from twenty unrelated Cypriot families and include two additional cases of Russian descent. All of these findings have extensively been published by the MGFT team in peer-reviewed scientific journals [12, 51-53]. It is important to clarify the specific *RET* pathogenic variants identified within these families. Among the 22 unrelated families, twelve patients (probands) carried the severe p.Cys618Arg pathogenic variant (54.5%). Additionally, two carried the p.Cys634Tyr (9.1%). Four patients harbored mutations, somatic delE632_L633, p.Val804Met, p.Ile852Met, and p.Arg886Gln, respectively (4.76% each). Three patients (13.7%) from these families had the p.Met918Thr mutation, which is associated with MEN2B (Fig. 7.3A). In a recent study conducted by the MGFT Department, there was evidence suggesting a founder effect for the frequently identified p.Cys618Arg pathogenic variant in the *RET* proto-oncogene. This pathogenic variant was found in twelve unrelated Cypriot families, suggesting a common ancestor [12].

To investigate the possibility of a shared origin for the p.Cys618Arg mutation, we employed a specific technique called "haplotype analysis" using microsatellite markers. This analysis revealed a common genetic signature (core haplotype) shared by all nine initial probands and their family members carrying the p.Cys618Arg pathogenic variant [12]. These findings strongly suggest that the p.Cys618Arg is an ancestral mutation. This could mean that it likely appeared in a single individual in the distant past and then spread throughout the Cypriot population through a founder effect (Fig. 7.3B). In patients with MEN2, the severity of MTC appears to be influenced by two key factors. The first is the presence of pheochromocytoma, a hormonal tumor that can indicate a more aggressive form of MTC. The second factor is the specific type of pathogenic variant that is inherited in the *RET* proto-oncogene. Since MEN2 is caused by mutations in this gene, the severity of the mutation itself can influence how aggressive the MTC becomes [46]. Fortunately, for patients with MEN2, early detection and preventive thyroidectomy can successfully prevent the development of malignant Medullary Thyroid Carcinoma (MTC). This surgery typically occurs after diagnosis or before the age at which MTC is likely to become cancerous [46]. In line with current best practices, most Cypriot MEN2 patients and their at-risk relatives identified with serious *RET* pathogenic variants have

undergone preventive total thyroidectomy with central lymph node dissection. Unfortunately, this wasn't always the case and some individuals with a delayed diagnosis passed away [12]. For a deeper understanding of the *RET* proto-oncogene pathogenic variants linked to MEN2, two key resources can be explored: 1) The Leiden Open Variation Database (LOVD) (https://www.variome.info/), a comprehensive resource allowing you to search specific *RET* gene variants and find their prevalence and associated pathogenic effects on how they disrupt gene function and contribute to the disease 2) The Revised American Thyroid Association (ATA) Guidelines, which provide up-to-date recommendations for managing MTC based on the latest research on *RET* pathogenic variants. These guidelines, likely available on the official ATA website, offer valuable insights for diagnosis, risk stratification, therefore determining disease severity based on mutation type, and treatment strategies [46].

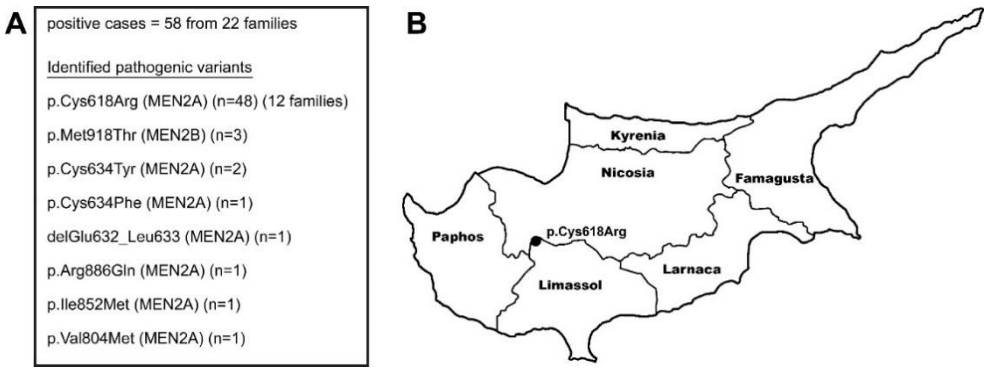

Fig. 7.3 (A) The pathogenic variants of the *RET* proto-oncogene identified in the MGFT department of the Cyprus Institute of Neurology and Genetics. 58 cases sharing an autosomal dominant pathogenic variant in the *RET* proto-oncogene were identified. Among the 22 unrelated families, twelve patients (probands) carried the severe pathogenic variant p.Cys618Arg. **(B)** Family histories of five of the MEN2A families with the p.Cys618Arg mutation reveal that they originated from the same village in the northwestern end of the Limassol district in Cyprus.

7.2.3 Using Genetic Testing to Identify Causes of Pubertal Timing Disorders

Over 400 tests have been conducted to date, utilizing a range of advanced techniques like Next-Generation Sequencing (NGS), Sanger sequencing, and multiplex ligation-dependent probe amplification (MLPA) for diagnosing early or late puberty. These tests aim to identify potential genetic factors that may contribute to these pubertal timing disorders.

7.2.3.1 Precocious Puberty

Precocious puberty, or early puberty, is when a child's body develops adult physical characteristics too soon, typically before age 8 in girls and 9 in boys [54]. In the most common form, central precocious puberty (CPP), this happens because the hypothalamus in the brain starts releasing gonadotropin-releasing hormone (GnRH) too early [55, 56]. This GnRH triggers the pituitary gland to release other hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then signal the ovaries in females or testes in males to produce sex hormones, leading to sexual development. There is also a less common peripheral form of precocious puberty caused by sex hormone production from other sources such as the adrenal glands or even tumors [54, 57]. This can lead to breast development, pubic hair growth, rapid growth, and even menstruation in girls or testicular enlargement and voice deepening in boys. While not always medically concerning, precocious puberty can cause emotional and social problems for children due to early physical changes. It is therefore imperative to seek medical advice from a pediatric endocrinologist for diagnosis and discuss treatment possibilities if needed.

In a collaborative effort researchers and doctors from CING and external clinicians collaborators have identified the *MKRN3* gene as a critical factor in CPP, particularly in females of Cypriot ancestry [4, 5, 15, 16, 31]. The intronless *MKRN3* gene (Fig. 7.4) plays a vital role in regulating puberty timing and loss-of-function pathogenic variants that are paternally inherited are the leading cause of CPP mostly in girls [58, 59]. As of today, a large group of >200 female patients of Cypriot origin clinically diagnosed with CPP has extensively been investigated by MGFT Department at CING. The identified specific pathogenic variants in the *MKRN3* gene were not just limited to the known protein-coding region of the gene, but also found in the promoter and 5'-UTR regions [4, 15, 17, 18]. Interestingly, nine girls from seven nonrelated families of Cypriot descent all shared the same novel loss-of-function p.Gly312Asp pathogenic variant, indicating another possible founder effect phenomenon [15, 18]. The study also revealed interesting findings in a specific subset of the girls. Eight girls with CPP carried mutations in the *MKRN3* gene that are likely to cause the condition. These mutations were located upstream of the gene's coding region, suggesting that they might influence gene expression rather than protein structure directly. Specifically, four girls had variations named *rs139233681* and *rs74005577* in the promoter region before the transcription start site. One girl had a variation named *rs131589420* in the promoter, and another had a variation named *rs184950120* in the 5' untranslated region (5'-UTR) [4, 18]. For all girls with CPP who underwent genetic testing at the CING and were found to have disease-causing pathogenic variants in the coding region, promoter, or 5'-UTR of the *MKRN3* gene, a confirmation test was performed. This test measured their levels of LH after stimulation with GnRH. If confirmed, these girls received treatment with a long-acting GnRH analogue medication, which effectively reversed the signs of puberty [4, 15, 17].

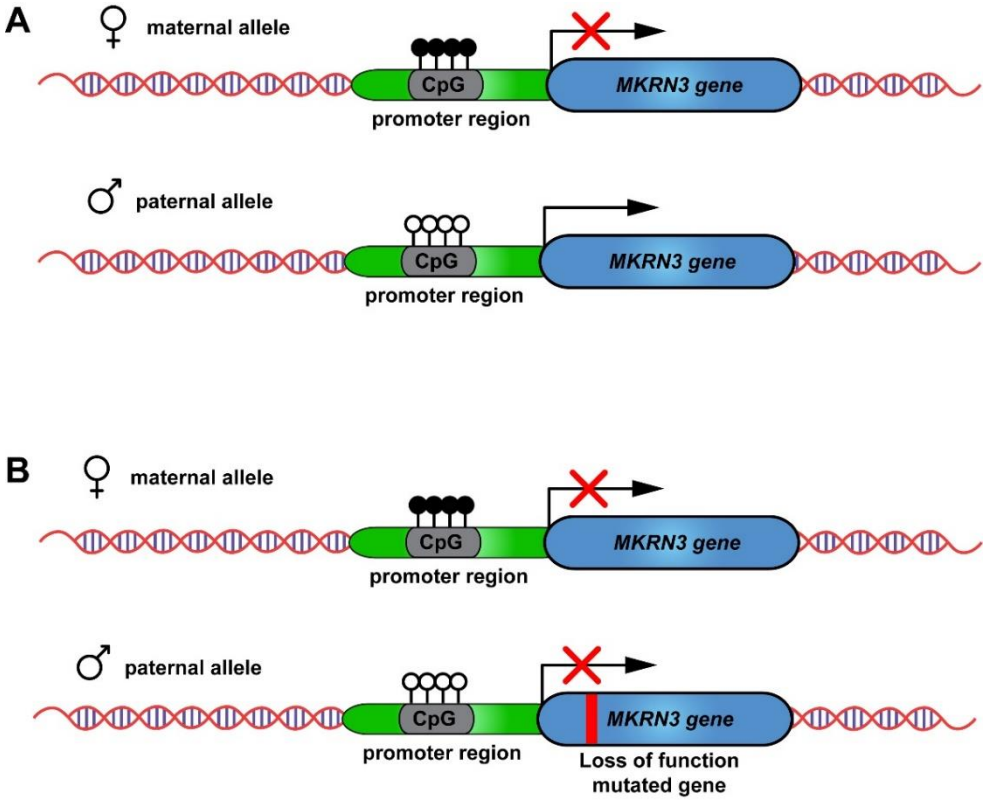

Fig. 7.4 (A) Schematic representation of normal imprinting of the *MKRN3* gene with silencing of the maternal allele by methylation of the CpG island in the promoter region and expression of the paternal allele. (B) Representation of paternal inherited loss-of-function mutation. Both alleles are inactive since the paternal is mutated and the maternal is silenced.

To explore other potential causes, whole exome sequencing (WES) by NGS was performed on a subgroup of 44 girls who didn't have *MKRN3* mutations. The findings from the WES analyses were recently published in a peer-reviewed journal, highlighting the potential role of rare variations in *DLK1*, *KISS1* and *MAGEL2* genes in contributing to CPP in some cases, particularly for those who don't have *MKRN3* pathogenic variants [18].

Beyond genetic factors like imprinting, puberty is also influenced by epigenetics, which involves other mechanisms such as DNA methylation at CpG dinucleotides in puberty-related genes. Researchers from the MGFT Department at CING recently investigated DNA methylation, a specific epigenetic mechanism, in the *MKRN3* gene promoter of the mouse hypothalamus. The results obtained from this study indicated that methylation levels at a cluster of CpG dinucleotides (CpG islet) within the promoter were significantly lower before puberty compared to pubertal and post-

pubertal stages. This suggests that DNA methylation in this region might play a role in regulating the timing of puberty onset [5]. The same study also employed computational analysis (in silico analysis) to explore the transcription binding sites on the *MKRN3* CpG islet. This analysis identified 29 potential regulators that bind to DNA in this region, with 14 of them acting as repressors [5]. These findings suggest that the *MKRN3* promoter might be regulated by a complex interplay of activators and repressors that bind to the CpG islet, potentially influencing gene expression and puberty timing. At present, other in vitro studies by MGFT are underway aiming to delineate the possible mechanisms and the consequences of differential methylation of the *Mkrn3* promoter.

7.2.3.2 Delayed Puberty

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic condition that disrupts normal pubertal development and fertility [60, 61]. Eight individuals (six males and two females) diagnosed with CHH underwent whole exome sequencing (WES) using next-generation sequencing (NGS) at the CING's MGFT department. The WES results revealed mutations in genes previously linked to CHH, Kallmann syndrome which is a specific form of CHH characterized by anosmia, and delayed puberty [13]. The study revealed nine disease-causing pathogenic variants. Seven of these pathogenic variants were novel (not previously reported), while the remaining two had been identified in prior studies [13]. Interestingly, for the newly discovered pathogenic variants various computational tools were used to analyze their impact might have on the proteins produced by their specific genes. Following this in silico analysis, it was conceived through structural analysis how these pathogenic variants could disrupt protein function at a more detailed level. The confirmation that these mutations had the potential to disrupt protein function strongly suggests they are likely the reason causing CHH in these patients [13]. Therefore, by uncovering in Cypriot CHH patients a series novel pathogenic variant, this study paves the way for future research that could lead to more accurate diagnostic tools and potentially even new treatments for CHH and similar diseases.

7.2.4 Pathogenic Variants causing Disorders of Sexual Differentiation

The MGFT Department has extensive experience diagnosing disorders of sexual development. For over a decade, the Department offered DNA testing using both Sanger sequencing and the more advanced NGS technology for children born with ambiguous genitalia and adults with genital abnormalities. Such cases include the rare disorder of sexual development called 5α reductase deficiency [62, 63]. This condition affects individuals with XY chromosomes (typically male) who are born with ambiguous genitalia. The MGFT department identified this condition in five unrelated Cypriot females who underwent genetic screening after birth. The genetic

test focused on the *SRD5A2* gene, known to play a role in 5α reductase deficiency, and revealed specific disease-causing mutations (pathogenic variants) in these patients [7, 64]. The same study by Skordis et al. [64] suggests that the unusually high prevalence (almost 1%) of the *IVS1-2A>G* genetic mutation in Cypriots might be due to a founder effect. The MGFT Department previously reported on two unrelated newborns (one Cypriot and one Greek) with a rare genetic condition called 17β-HSD-3 deficiency. This was back in 2012 and 2018, respectively. Genetic testing confirmed the diagnosis in both cases. They inherited a combination (compound heterozygosity) of new and previously known disease-causing mutations (pathogenic variants) in the *HSD17B3* gene. Following their genetic diagnosis, both newborns underwent successful surgeries to correct cryptorchidism (undescended testicles) and hypospadias (abnormal positioning of the urinary opening) [8, 9].

7.2.5 Genetics of Glucose and Insulin Homeostasis – Maturity Onset Diabetes of the Young and Obesity

Molecular diagnosis of glucose and insulin homeostasis utilizes advanced techniques to analyze genes, proteins, and metabolites involved in blood sugar control. This approach offers earlier detection of diabetes risk, improved diagnosis of different types, and the potential for personalized medicine by identifying the underlying molecular cause and guiding treatment plans.

CING's MGFT Department offers a comprehensive molecular analysis to investigate defects in patients with glucose and insulin control problems. This includes identifying genetic causes of conditions like Maturity Onset Diabetes of the Young (MODY) and obesity linked to melanocortin-4 receptor (MC4R) deficiency. Their in-depth analysis utilizes a computer-based panel that examines 58 genes (*ABCC8 ADCY3 AGPAT2 ALMS1 BLK BSCL2 CEL CISD2 DCAF17 DMXL2 EIF2AK3 FOXC2 FOXP3 FTO GATA4 GATA6 GCK GLIS3 HAMP HFE HJV HNF1A HNF1B HNF4A IER3IP1 IL2RA INS INSR KCNJ11 KLF11 LMNA LRBA MC4R MNX1 MT-TL1 NEUROD1 NEUROG3 NKX2-2 PAX4 PCBD1 PDX1 PIK3R1 PLIN1 POLD1 PPARG PTF1A RFX6 SLC19A2 SLC29A3 SLC2A2 SLC40A1 STAT1 STAT3 TFR2 TRMT10A WFS1 ZBTB20 ZFP57*) known to be linked with the clinical phenotype of glucose and insulin homeostasis. The ongoing genetic study by the MGFT Department has so far analyzed over 50 patients from public hospitals and private medical centers in Cyprus (Archbishop Makarios III, Larnaca General Hospital, Limassol General Hospital, Paedi Medical Center, and Aretaeio Private Hospital) for mutations in genes known to be affecting glucose and insulin control. These patients, of various ages from newborns to adults, all displayed clinical and biochemical signs of problems with blood sugar regulation. The study so far identified 4 patients with MODY type 2 caused by mutations in the *GCK* gene, and 2 patients with MODY 3 caused by mutations in the *HNF1α* gene. These patients were diagnosed with diabetes between 10-28 years old and had average fasting blood

sugar levels slightly higher than normal. An oral glucose tolerance test showed an increase in blood sugar after 2 hours, but their insulin levels also rose, suggesting their bodies were still producing some insulin.

In a recent study by the MGFT Department, the role of *ADCY3* gene variants in regulating body weight was explored. The study included 33 severely obese adolescents and young adults aged 15-20 years (18 females and 15 males)[20]. All patients had a Body Mass Index (BMI) > +2.5 standard deviations (SDS) above average at the time of genetic testing and had a confirmed diagnosis of early-onset obesity defined by a BMI > +2 SDS from the age of 3 years onwards, and their medical records showed no presence of any other underlying medical conditions. The study also included a control group of 51 age-matched, non-obese individuals (40 females, 11 males) specifically chosen from the Greek-Cypriot population. Genetic analysis of the *ADCY3* gene in the patient group identified five variants, with four having been documented in previous studies. Among the five variants identified in the patients, one was novel and involved a single nucleotide change (c.349T>A) within exon 1 of the *ADCY3* gene. This change resulted in a substitution of the amino acid leucine (Leu) with methionine (Met) at position 117 of the protein (p.Leu117Met). Interestingly, this novel variant was found in two non-syngeneic patients. Given the rarity of monogenic obesity, the identification of these variants in our study of patients with severe childhood obesity strengthens the case for ADCY3 playing a significant role in regulating energy balance and potentially contributing to obesity development.

7.3 Conclusions

This chapter summarizes the findings of the past fifteen years at the MGFT Department, an ENDO-ERN Reference Centre within the Cyprus Institute of Neurology and Genetics. It highlights the critical role of advanced diagnostic techniques and research efforts in understanding inherited endocrine diseases. These advancements align with the core mission of the European Reference Networks: to facilitate access to accurate diagnoses, pave the way for targeted treatment strategies, and ultimately achieve unified management plans for these complex conditions.

Acknowledgments The Department of Molecular Genetics, Function and Therapy of the Cyprus Institute of Neurology and Genetics would like to thank the European Reference Network on Rare Endocrine Conditions: Project ID N0 739543 (https://endo-ern.eu/about/reference-centres/ accessed on 24th of September 2024).

Funding: This work was supported by the A.G Leventis Foundation.

Declarations

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and Cyprus National Ethics Research Committee (ΕΕΒΚ/ΕΠ/2016/28) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for Publication

Informed was obtained from all adult participants and the participants' legal guardians/next of kin.
Competing Interests The authors declare no competing interests.

Data availability

As this report summarizes previously published work by our group, data sharing is not applicable in this instance.

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Chapter 8
The Biobank of Cyprus
New Prospects for Next Generation Biomedical Research in Cyprus

Constantinos Deltas, Gregory Papagregoriou, Apostolos Malatras, Dionysios Fanidis and Mariel Voutounou

Cite this chapter:

Deltas, C., Papagregoriou, G., Malatras, A., Fanidis, D. and Voutounou, M. (2025). The Biobank of Cyprus: New Prospects for Next Generation Biomedical Research in Cyprus. In: A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 207-236). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 8

The Biobank of Cyprus

New Prospects for Next Generation Biomedical Research in Cyprus

Constantinos Deltas[1,2], Gregory Papagregoriou[2], Apostolos Malatras[2], Dionysios Fanidis[2] and Mariel Voutounou[2]

1: School of Medicine, University of Cyprus, Nicosia, Cyprus

2: biobank.cy Center of Excellence in Biobanking and Biomedical Research, University of Cyprus, Nicosia, Cyprus

Keywords: Biobank, Research infrastructure, Big Data, Biological material, Precision medi-cine, Bioethics

Chapter Abstract Biobanks are organized collections of medical records and biological material, including DNA, aimed at enabling precision medicine. Serving as research infrastructures, Biobanks provide high-quality data and materials for clinical trials, epidemiological studies, and genetic research. They facilitate the discovery of new biomarkers to achieve accurate diagnosis, prognosis, and disease prevention, as well as the discovery of innovative disease-specific therapeutics. The archived data and materials represent a national treasure accumulating added value over time, available to Cypriot researchers and the health services, enhancing citizens' quality of life and saving public money. As a key pillar of the Centre of Excellence for Biobanking and Biomedical Research, the Cyprus Biobank fosters collaborations amongst the scientific community and volunteers (patients and healthy individuals), contributing to an all-round improvement of life quality for everyone. Achieving this vision relies on the altruistic participation of thousands, who, instead of remaining passive observers, are encouraged to become citizen-scientists, joining the country's most ambitious medical project. This project, CY-Biobank, co-funded by the European Commission, the Republic of Cyprus, and the University of Cyprus, collaborates with the European Biobank Consortium BBMRI-ERIC, the Medical University of Graz, and the SME RTD Talos Ltd, in Nicosia. Our motto is: We invest in Biobanking, we invest in a healthier Cyprus.

8.1 Introduction

Following funding to create a Centre of Excellence in Research and Innovation, focused on Biobanking and Biomedical Research in Cyprus, an inaugural event was held at the University of Cyprus on November 26-27, 2019. This event presented the planned actions and strategies for implementing the project. The initiative stems from a scientific proposal submitted to the European Commission under the H2020-WIDESPREAD-01-2018-2019: TEAMING program, which falls under the Coordination and Support Action (CSA) category. In pursuing its aim to promote balanced development across the European Union, the European Commission introduced the WIDESPREAD measure within the Horizon 2020 Program (2014-2015) for 15 out of 28 EU member states that demonstrate low research and innovation funding absorption. Recognizing the competitive disparity between newer and less developed member states and more established ones, the European Commission designed WIDESPREAD as a targeted initiative to advance research and innovation in these 15 *WIDENING* countries.

Under this measure, the European Commission funds each project with up to €15 million, with the proposing member state expected to match the funding. The proposals are intended to create research infrastructures comparable to those in developed EU states, aligning with each country's Smart Specialization Strategy and enabling better competitiveness. Thus far, through rigorous two-phase evaluations in three rounds, 40 projects in 15 WIDENING countries have been funded through WIDESPREAD. Cyprus has succeeded in obtaining seven projects, four coordinated by the University of Cyprus, one of which is described herewith, titled: *Biobanking and the Cyprus Human Genome Project (short name: CY-Biobank)*.

At the heart of the project is the upgrading of the Biobank, which was created previously with separate funding, and the increase in research infrastructure capacity to serve the Cypriot and global patients. Biobanks are medical research infrastructures that represent one of ten ideas changing the world, according to the 23 March 2009 Annual Special Issue of the Times Magazine.[1] They are infrastructures that systematically enrol citizens, healthy or diseased, who altruistically volunteer to participate by sharing personal, demographic and health data associated with biological material to be used in biomedical research. Confidentiality and high security measures need to be implemented to gain the trust of the public, in accordance with respect to the ethical, legal and social implications (ELSI). Data and material in the form of DNA, or else, are available in hundreds of Biobanks around the world and enable studies in all medical fields including genetics, epidemiology, biostatistics, biomarker discovery and drug development, to name a few, aimed at better disease

[1] 10 Ideas Changing the World Right Now: Biobanks
March 23, 2009 | Vol. 173 No. 1: https://content.time.com/time/specials/packages/article/0,28804,1884779_1884782_1884766,00.html

diagnosis, prognosis and prevention.

In summary, the *CY*-Biobank project entails creating a Centre of Excellence in Biobanking and Biomedical Research. At its core, the Biobank is a high-standard resource available to the Cypriot research community under strict privacy protections in compliance with the General Data Protection Regulation (GDPR, 2016/679). The Biobank is equipped with advanced research equipment, and tools that facilitate next-generation biomedical research in Cyprus, and develops innovative research activities after obtaining the necessary approvals from the Cyprus National Bioethics Committee (CNBC) for each individual project.

8.2 Project Coordination

Coordinator: Professor Constantinos Deltas | University of Cyprus Medical School

Project Title: Centre of Excellence – Biobanking and the Cyprus Human Genome Project (***CY*-Biobank**)

Grant Agreement Number: 857122 | **https://biobank.cy/**

Program: H2020-WIDESPREAD-01-2018-2019: Teaming Phase-2

Project Start Date: October 1, 2019

Total Project Budget:

- European Commission: €15 million
- Republic of Cyprus: €15 million
- University of Cyprus: €8 million

8.3 Collaborating Partners

1. University of Cyprus, Nicosia, Cyprus
2. Medical University of Graz / BBMRI.at, Graz, Austria
3. Biobanking and BioMolecular Resources Research Infrastructure - European Research Infrastructure Consortium / BBMRI-ERIC, Graz, Austria
4. RTD Talos Ltd, Nicosia, Cyprus

A budget of €30 million was foreseen for the first 7 years, now extended to 8 as justified by delays conferred during the COVID-19 pandemic. This funding will be invested, inter alia, for the creation of appropriate building infrastructure, the purchase of the necessary logistical-technical equipment, and the recruitment of the team (scientific and administrative) that will implement the project. Another €8

million have been budgeted to be invested during the subsequent 8 years (€1m per year), thus giving a 16-year sustainability horizon, according to the business plan submitted. It goes without saying that there is a continuous effort to secure further funding to ensure the long-term viability of the Centre, either through the development and offering of services to medical doctors and their patients or by obtaining further funding for research projects.

8.3.1 Description of Partners

University of Cyprus, Center of Excellence in Biobanking and Biomedical Research (short name biobank.cy): The Center biobank.cy, is one of the Centers of Excellence in Research and Innovation hosted by the University of Cyprus, and it represents the evolution of the Molecular Medicine Research Center (MMRC). MMRC was an independent research unit of the University of Cyprus that was previously created with €2 million in competitive funding by the Republic of Cyprus and the European Regional Development Fund through the Cyprus Research and Innovation Foundation (former Research Promotion Foundation) (Programme: Strategic Infrastructure Project: NEW INFRASTRUCTURE/STRATEGIC/0308/24). This proposal was ranked first amongst 42 proposals that were submitted and evaluated in a two-step process. Over the past five years, with the funding obtained through the *CY*-Biobank project, the MMRC was substantially upgraded and expanded, with the development of five pillars of activity (Fig. 8.1 and https://biobank.cy/):

Pillar 1 - Biobank: It is the first Biobank in Cyprus. It was created in 2011, as part of the objectives of MMRC. It offered for the first time the opportunity for the creation of proper registries and archiving of biological material associated with data to be used in research. Importantly, researchers from any research institutions in Cyprus, are offered an infrastructure for supporting their research, when biobanking and relevant support activities are needed, thereby empowering them to pursue their research endeavours, in a secure and GDPR-compliant environment. Having access to a high-standard Biobank infrastructure saves time and money for the researchers as they have the opportunity to seek data and material already available, or they can exploit the Biobank facilities and personnel expertise to carry out tasks they are not experts for or do not have the necessary funding to implement. Depending on the individual circumstances, the Biobank, per its mandate and upon the approval of the Cyprus National Bioethics Committee, is entitled to charge for its services based on cost-recovery to maintain its basic operation. Fees may differ when the entities are from academia versus the industry. A brief tabulation of the material biobanked thus far is available in Table 8.1.

Pilar 2 - Molecular Medicine Research Center (MMRC): Now maintained as a separate Pillar, MMRC started as a research unit at the UCY and evolved to

represent the research arm of the Center of Excellence focused on investigating human genetic diseases. Translational research projects on kidney disease, heart disease, autoimmune diseases and others are ongoing, as well as basic research projects and preclinical studies in mouse and zebrafish models. Indicative publications of previous research work at MMRC are the following: (Voskarides, Damianou et al. 2007, Gale, de Jorge et al. 2010, Athanasiou, Voskarides et al. 2011, Deltas, Pierides et al. 2013, Pieri, Stefanou et al. 2014, Deltas, Savva et al. 2015, Odiatis, Savva et al. 2021).

Over the years, research results of our group had substantial impact on the local and international nephrology front, either by implementing new diagnostic services or by contributing to a better understanding of disease mechanisms in inherited kidney conditions. Reportedly, our group was part of the original consortium that had mapped and then cloned the *PKD2* gene, pathogenic variants of which cause the autosomal dominant polycystic kidney disease type 2 (Mochizuki, Wu et al. 1996, Deltas 2001). Subsequently, we were the first to describe the trans-heterozygous mechanism for cyst formation in the affected kidneys of patients(Koptides, Hadjimichael et al. 1999, Koptides, Mean et al. 2000). According to this hypothesis, a patient inherits one mutant allele of the *PKD2* gene and then a second postzygotic hit inactivates the normal *PKD2* allele that was inherited from the healthy parent, thus leaving the cell with no normal *PKD2* function. In the trans-heterozygous mechanism for cyst formation, we showed that the second loss-of-function hit sometimes affects the healthy allele of the same gene (say the *PKD1* or the *PKD2*) or one allele of the other gene, thereby leading to a molecular situation where one *PKD1* or *PKD2* allele was the inherited mutant and the postzygotic somatic second hit independendly inactivated one allele of the other *PKD* gene, in the same cell.

Other impactful research projects of our group were:

- The mapping of the *MUC1* gene (formerly *MCKD1*). Mutations in this gene cause one of the ADTKDs (autosomal dominant tubule-interstitial kidney diseases), better known as MUC1 kidney disease. While it is rare in the rest of the world, it is highly prevalent in and around the prefecture of Pafos, south-west of the island of Cyprus, affecting approximately 1/600 people(Christodoulou, Tsingis et al. 1998, Stavrou, Koptides et al. 2002).

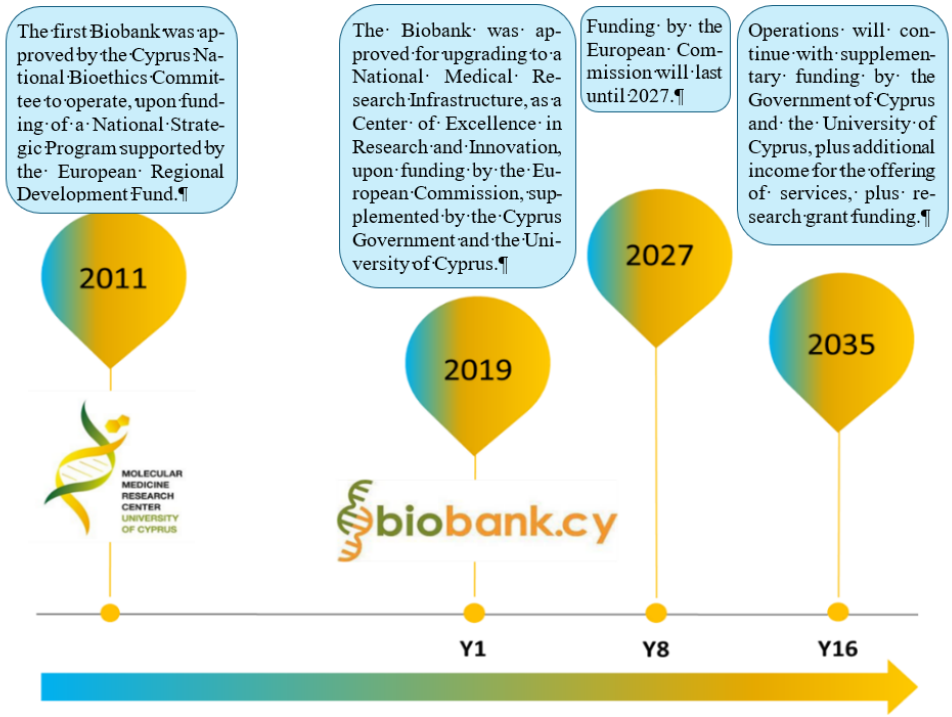

Fig. 8.1 Timeline showing the major steps in the history of the Biobank

The gene was cloned by a group at the Broad Institute of Harvard and MIT, Cambridge, Massachusetts, USA, in 2013 (Kirby, Gnirke et al. 2013). Subsequently, a collaboration started between the Broad Institute group and our group in Cyprus, through the Biobank, aimed to better understanding of the disease clinical course and preparing a cohort of Cypriot patients for future clinical trials (Bleyer, Kmoch et al. 2014)(Papagregoriou G et al, *Unpublished results*).

- We were the first to demonstrate the activation of the unfolded protein response (UPR) as part of the mechanism of development of Alport syndrome, a severe renal glomerulopathy (Pieri, Stefanou et al. 2014, Odiatis, Savva et al. 2021). This discovery paved the way for testing novel treatments with repurposed and new drugs.
- We were the first to create a mouse model for Alport syndrome that carries a glycine substitution mutation in the *COL4A3* gene, rather than a knockout collagen IV[2] mutation, as all previous similar models. The mutation we introduced in the

[2] *COL4A3* and *COL4A4* and *COL4A5* (X-linked gene) are genes which encode the α3, α4 and α5 chains of triple helical collagen IV protomer molecules. Trimeric α3α4α5 collagen IV protomers constitute the most abundant molecules and are essential in forming the glomerular basement membrane in the kidney, a kind of specialised extracellular matrix.

mouse, at residue 1332 (p.Gly1332Glu) recapitulates the human counterpart, p.Gly1334Glu, which is the most prevalent founder variant in patients with Alport spectrum nephropathy in the Cypriot population[3]. This mouse model enabled us to perform studies that led to new insight regarding the molecular and cellular mechanisms of disease progression and gave the opportunity for preclinical trials with the use of repurposed approved drugs (Pieri, Stefanou et al. 2014, Odiatis, Savva et al. 2021).

- Finally, we were the first to provide a large volume of data to establish that patients who are heterozygous for collagen IV alpha3 or alpha4 pathogenic variants and present with familial microscopic hematuria because of thin basement membrane nephropathy, do not always follow a benign course with excellent prognosis, as previously thought. Instead, these patients have a life-long variable risk of progressing to clinically significant proteinuria and chronic kidney disease, even end-stage renal disease later in their life, at an average age of 56-years (Voskarides, Damianou et al. 2007, Pierides, Voskarides et al. 2009, Deltas, Pierides et al. 2012, Deltas, Pierides et al. 2013, Deltas, Savva et al. 2015).

Pillar 3 - Laboratory of Molecular Diagnostics: The development and provision of molecular diagnostic services in Cyprus and abroad has been our activity for many years with the Ministry of Health as the main recipient. The laboratory is a referral center for rare monogenic kidney diseases, both in Cyprus and Greece, including its well curated archive and renowned expertise (Deltas, Gale et al. 2013). Since the establishment of the laboratory, in a systematic manner, we expanded our services for the first time in Cyprus into the field of monogenic inherited cardiomyopathies, initially in close collaboration with Dr Panayiotis Avraamides and Dr Marios Ioannides, Head and Deputy Head respectively, of the Department of Cardiology of the Nicosia General Hospital, to develop and offer a molecular diagnostic pipeline that served more than 200 families to date (Apostolou, Ioannides et al. 2023, Koutsofti, Ioannides et al. 2024).

Pillar 4 - Education Pillar: Researchers of the Centre gave dozens of lectures at national and regional conferences, in public hospitals and other medical centers in Cyprus and abroad for educational purposes on molecular and medical genetics. In a targeted manner, lectures have been given in departments of nephrology, pediatrics and cardiology, while in the context of the operation of *CY*-Biobank, training sessions for doctors and medical students are offered regularly to promote Biobanking.

Pathogenic variants in these genes cause the classical Alport syndrome and variants of it currently referred to as Alport spectrum nephropathy. Patients heterozygous for *COL4A3* or *COL4A4* variants were previously diagnosed with thin basement membrane nephropathy, a term which tends to be annulled.

[3] For more information about founder populations and founder mutations in the Cypriot population, the reader is referred to a book published by Prof. C. Deltas entitled: *The genetic heritage of Cypriots through special topics of genetics*. BHTA Medical Arts, Athens 2015; ISBN: 978-960-452-180-7 (Greek)

Our Biobanking operation has been recognised not only in Cyprus but also abroad, and several entities sought to visit us to learn from our experience in setting up a new Biobank. Also, students and other scientists from foreign Universities received training in our setting through educational ERASMUS+ programs or based on bipartite arrangements. Notably, members of the Center actively participate in delivering modules for the post-graduate degree program MSc in Biobanking, of the Medical University of Graz, Austria.

Pillar 5 - Innovation pillar: Innovative projects aimed to solve important health problems are in progress. These pertain to a better understanding of the clinical course of diseases and the molecular mechanisms of disease development as well as attempting innovative treatments in mice models of human disease. Additionally, experimentation in more pioneering research areas concerns the development of nanoparticles for more effective drug delivery and organoids construction as alternatives to using animals for experimental research.

8.3.2 Other external Partners

BBMRI-ERIC (https://www.bbmri-eric.eu/): With Director General Prof. Jens Habermann, this is the largest Consortium of European Biobanks. Twenty-two countries participate and regular meetings are held to discuss common problems and goals pertaining to achieving high-quality biobanking and effective collaboration among members and observers. The Consortium's priority sectors include the harmonization of activities of biobanks, the definition of communication protocols, and establishment of curated standards for high quality medical data and biological material they manage. Also, a major objective is the promotion of cutting edge research stemming out of biobanks themselves, with respect to the ethical, legal and social issues emanating from the biobanking operation. Cyprus joined BBMRI-ERIC with an Observer status in 2016, and then became a full member in 2020 on our initiative, as a set requirement of the *CY*-Biobank project.

Medical University of Graz / BBMRI.at (http://bbmri.at/): The MUG hosts the largest cancer biobank infrastructure in Europe, which was awarded in 2014 as the best European Biobank. Prof. Zatloukal of MUG, and current Director of the Austrian Biobanks national node, was the first to launch in 2008, with funding from the European FP7 program, the initiative to promote the idea of harmonizing Europe's Biobanks and improving the quality of services they offer to researchers.

Both BBMRI-ERIC and MUG are serving as Advanced Partners in the project, per the instructions of the Horizon 2020 call that required the collaboration of the applicants with relevant expert groups in other countries that could offer guidance and expertise in implementing the proposed research infrastructures. These partners were highly cooperative and proved instrumental in establishing contemporary Biobanking procedures administratively and scientifically, implementing quality and security measures and obtaining certification for ISO9001:2015 while respecting

ELSI issues. For compliance with the GDPR and for preparing our Data Protection Impact Assessment we had to collaborate with external legal professionals in Cyprus.

RTD Talos LTD (http://www.talos-rtd.com/): Managed by Dr Alexandros Michaelides, RTD Talos is a small-sized enterprise operating in Cyprus and abroad and has the role of coordinating and promoting activities for dissemination and sustainability of the *CY*-Biobank project. Talos was of the greatest assistance since the beginning with the preparation and submission of the proposal, mainly on technical and administrative issues and contributes to issues related to administration and business development.

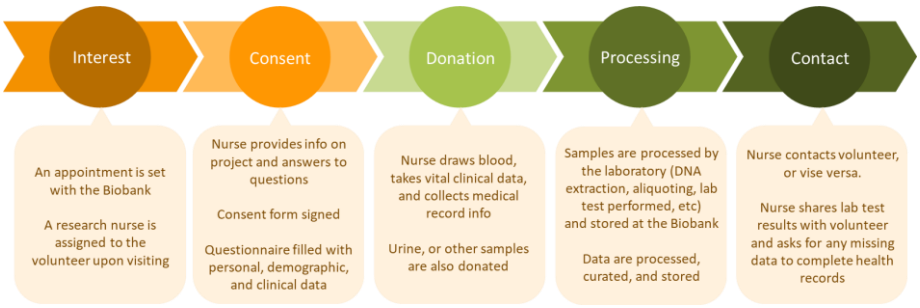

Fig. 8.2 Major steps involved in biobanking enrolment.

8.4 Project Objectives

The general objectives of the biobank.cy Center of Excellence, are as follows:

1. **Strengthening the Biobank of the Centre of Excellence** through expanded activities to cover many genetic and other diseases, whether common or rare. Diseases being served include:
   - **1.1. Chronic kidney diseases (CKD):** Study of inherited monogenic and multifactorial kidney diseases, with the consideration that Cyprus ranks first with the highest incidence of patients developing kidney failure amongst the EU countries (Boenink, Astley et al. 2022). Also, CKD manifestation itself confers a much higher risk to patients for developing and perhaps dying of a heart incident before even they reach the stage of kidney failure.To this grave statistic, there are data according to which the genetic heritage of Cypriots has a great contribution. It is not clear yet to what extent genetics and environmental insults play a role, and this is a field where experimental solid evidence must be derived. Hence, deciphering the genetic predisposition of Cypriots to developing all types of CKD is a prime goal of the Center, and is set to assist in future epidemiological studies and public policy measures to curb this situation.

Research in our laboratory during the past more than 30 years, initially collaborating with Dr Alkis Pierides, Head of Nephrology at the Nicosia General Hospital and subsequently with the entire clinical nephrology community in Cyprus, revealed a heavy burden of inherited diseases leading to CKD. These include polycystic kidney disease, the most common inherited renal condition worldwide, but also MUC1 kidney disease, and hereditary hematurias, as well as other rare conditions. Our work through the years demonstrated that a substantial percentage of patients who inherit heterozygous pathogenic variants in the collagen-IV *COL4A3* and *COL4A4* genes do not follow a benign course but on long follow-up develop added proteinuria and severe CKD, even kidney failure, requiring renal replacement therapy or a kidney transplant. More recent work in our laboratory has attempted to investigate the hypothesis that patients sharing the same or similar pathogenic variants explaining their disease have a higher predispositon to progressive CKD due to the incidental co-inheritance of additional DNA variants in single or multiple genes, namely genetic modifiers, which aggravate the phenotype. To test this hypothesis in a systematic way, we performed whole exome sequencing on many patients classified either as being *mildly* or *severely* affected, to define the burden imposed by their individual genetic profiles towards the severity of their phenotype. We are currently in the process of diligently analysing these data.

– **1.2. Inherited heart conditions**: The leading cause of death in the Cypriot population is cardiovascular disease, and this fact opted us to prepare a unique registry of families presenting with heart diseases, starting from inherited cardiomyopathies. The creation of this cohort aims to empower clinicians and researchers together to disentangle seemingly disguised inheritance patterns responsible for heart conditions going beyond our current understanding, especially concerning the genetic basis of sudden cardiac death. Our goal is to eventually convince local authorities to establish genetic screening pipelines as parts of forensic investigations for sudden deaths in the future, and in extend advice family members. The Center currently offers a comprehensive genetic diagnostic test panel for cardiomyopathies, while at the same time runs satellite research projects set to undersrand the molecular basis of this group of diseases. Hence, more than 70 relevant genes have already been screened in patients and relatives from nearly two hundred Cypriot cardiomyopathy families.

After the elucidation of the molecular defect in tens of patients, we have shaped an initial understanding of the genetic heterogeneity observed in the Cypriot population, with pathogenic variants which are either novel or already published in the international literature and spotted in the available mutation databases. Notably, the identification of variants with a wide presentation among different populations including Cypriots, can be explained by the continuous genetic flow caused by migrating populations around the world, to

eventually enrinch local gene pools. Complying to international standards, genetic diagnostics at the Center evaluate and classify emerging variants by adopting the ACMG criteria (American College of Medical Genetics and Genomics and the Association for Molecular Pathology) (Richards, Aziz et al. 2015). Finally, in our zebrafish facility, animal models are being created for the functional characterization of identified viariants towards triggering diseases phenotypes, and as means to even evaluate novel and variant-specific treatments.

- **1.3. Eye diseases**: In collaboration with the Ophthalmos Research and Educational Institute, Nicosia, Cyprus, headed by Dr Tassos Georgiou, we are implementing a pioneering project for Cyprus, by actively enrolling patients with chronic eye conditions to facilitate current and future studies working on the genetic, clinical, and epidemiological assessment of chronic eye diseases, paving the way for implementing clinical trials. A registry of more than a thousand patients, at times including other family members as well, has been created and is being analysed genetically, through whole exome sequencing and bioinformatics. This registry enabled us to evaluate the prevalence of monogenic vision problems as an outcome of a single center. Moreover, the registry includes individuals diagnosed with multifactorial conditions or eye defects associated to physiological aging, such as glaucoma, keratoconus, retinal dystrophies, and others.
- **1.4. Cyprus COVID-19 project**: During the COVID-19 pandemic, in the framework of a research project, our Center was the first to measure the serum IgG antibody load of Cypriots against SARS-CoV-2 corona virus, and then study the epidemiological build-up of the pandemic in Cyprus by seeking for statistical relationships based on the participants' demographic and clinical data. The work was performed on 969 unvaccinated individuals, who were reportedly infected between November 2020 and September 2021. The antibody levels were generally higher in participants of older age, in males, and in those who reportedly developed symptoms or were hospitalized. The receptor binding domain (RBD)-specific IgG levels peaked at three months post symptom onset and subsequently decreased up to month six, with a slower decay thereafter. IgG response to the RBD of SARS-CoV-2 was bi-phasic with considerable titer variability. Levels of IgG were significantly associated with several parameters, including age, gender, and severity of symptoms (Mamais, Malatras et al. 2021). Whole Exome Sequencing of the participants is now underway, to study the contribution of genetic variants in the severity of accounted symptoms, in collaboration with the genetics center of Regeneron, a pharmaceutical company in New York.
- **1.5. Primary ciliary dyskinesia (PCD):** It is a rare genetic disorder characterized by defects in cilia structure and ineffective cilia function. The disease is highly heterogeneous, and the diagnosis is challenging, as there is no gold-

standard diagnostic test. It causes serious morbidity in young children, while permanent lung damage is highly likely early on. In collaboration with Prof. Panayiotis Yiallouros of the UCY Medical School, a registry is being prepared accompanied by genetic testing, in a systematic manner, thereby offering a genetic diagnosis while biobanking data/material for further research(Yiallouros, Kouis et al. 2021).

Table 8.1 Brief tabulation of the total participants enrolled as far in the Biobank, and the project in which they currently participate.

Project/Cohort Title	Number of Participants	Types of Samples Stored	Associated Data
***CY*-Biobank (General Population)**	4,162	DNA Serum Plasma, Sodium Citrate Plasma, EDTA Urine	Clinical Data Biometrics (Height, Weight, BMI, Oxygen levels, BP, HR etc.) Family History Data Lifestyle (diet, exercise, sleep) Hematological, Biochemical Data Whole Exome Sequencing completed for 1446 samples (CY-PROME_v3)
Ophthalmos (Eye-diseases cohort)	1,727		
CY-Nephron	900		
CY-COVID-19 (post-Covid19, post-vaccination, healthy participants)	2708		
CYGEN-Cardio (Cardiomyopathies cohort)	896	DNA	
β-thalassemia Cohort	120		
Cystic Fibrosis Cohort	60		
Total Participants Enrolled	**10,573**		

Additional biobanked cohorts under development are:

- **1.6. Systemic lupus erythematosus (SLE) - Coordination: Dr Konstantinos Parperis, Assistant Professor at the UCY Medical School:** A registry is being prepared with biospecimens and clinical data to generate several studies in extend. A case-control study is also in progress, set to identify genetic variants contributing to SLE phenotypes.
- **1.7. Peripheral Nervous System Involvement in Coeliac Disease - Coordination: Dr Panagiotis Zis, Assistant Professor at the UCY Medical School**: This project aims to investigate how coeliac disease affects the peripheral nervous system (PNS). It aims to identify specific neuropathic symptoms associated with gluten sensitivity and explore the underlying mechanisms. By analyzing nerve conduction studies and patient-reported outcomes, the study seeks to enhance diagnosis and management strategies for affected individuals. Understanding the involvement of PNS in coeliac disease , may in turn lead to improved therapeutic approaches.
- **1.8. Natural History and Phenotyping of Peripheral Neuropathies of Various Etiologies - Coordination: Dr Panagiotis Zis, Assistant Professor, UCY Medical School**: This project focuses on the diverse causes of peripheral neuropathies, including of diabetic, hereditary, and autoimmune origins. By collecting longitudinal data, the project aims to characterize the natural

progression of these conditions and identify distinct phenotypes. Utilizing advanced imaging and genetic analysis, the study will provide insights into disease mechanisms and potential therapeutic targets, ultimately improving patient outcomes and personalized treatment plans.

- **1.9. Sensory Perception in People with Neurodevelopmental Disorders - Coordination: Dr Panagiotis Zis, Assistant Professor, UCY Medical School**: This project will explore how neurodevelopmental disorders, such as autism spectrum disorders and ADHD, influence sensory perception. Through behavioural assessments and neuroimaging techniques, the project aims to identify atypical sensory processing patterns. By understanding these differences, the study seeks to inform interventions and support strategies that enhance sensory integration and improve daily functioning for individuals with neurodevelopmental challenges.
- **1.10. PrePARE for LIFE study - Coordination: Dr Ourania Kolokotroni, Assistant Professor, Cyprus University of Technology**: The study evaluates a variety of preconception exposures and their role in shaping health behaviours and outcomes. Primary volunteers are students at the nursing school, but also students from other departments have been invited to enrol.
- **1.11. Primary school children's health and environment observatory - A pilot child cohort study in Cyprus – Coordination: Prof. Konstantinos Makris, Cyprus University of Technology**: The study focuses on the investigation of the origins of non-communicable diseases (NCDs) during childhood, thereby allowing for cohort evidence-informed effective primary preventive interventions. This study will pay particular attention to the early stages of the NCD process, by focusing on targeted and untargeted biomarkers of effect, including inflammation, oxidative stress/damage, fatty liver and kidney function. This study is very much exploring the concept of the exposome, aimed at associating environmental exposure to health morbidities.

2. **Applying eHealth principles:** Working towards building an integrated health data ecosystem, we are implementing eHealth principles established by the European Commission, promoting a streamlined, comprehensive, and unified approach to health data creation and management. These include:

- Data interoperability, is pivotal for biobanks as they serve as central data hubs in biomedical research. Seamless collaboration and data exchange, both within biobanks and with external research institutions, require a robust framework built on key strategies. These include implementing standardization, leveraging standardized APIs and data integration tools, adopting robust metadata management practices, and aligning with the FAIR principles (Findable, Accessible, Interoperable, Reusable).
- Standardization, is fundamental to achieving data interoperability, as it harmonizes data to enable consistent and reliable integration. By adopting widely

accepted data standards such as HL7 (Health Level Seven), FHIR (Fast Healthcare Interoperability Resources), and DCAT-AP (Data Catalog Vocabulary Application Profile), biobanks can ensure compatibility and consistency across diverse systems, including hospitals, clinics, and genomics laboratories.

A. HL7 provides a suite of international standards for the exchange, integration, and retrieval of electronic health information, ensuring structured health data can be shared across various healthcare systems.
B. FHIR, developed by HL7, facilitates rapid and efficient exchange of healthcare information via RESTful APIs, supporting data formats like JSON and XML. It is particularly suited for handling diverse datasets, from simple clinical records to complex genomic data.

Our Biobank adopted these standards to ensure seamless integration of clinical data originating from disparate electronic health record (EHR) systems. This supports the Interoperable and Reusable principles of FAIR by maintaining consistent data formats usable across multiple research projects. Early-stage data mapping to these standards prevents inconsistencies stemming from diverse data formats. To further enhance standardization, we also use

A. Standardized ontologies such as SNOMED CT or LOINC for clinical data.
B. Provenance information standards, to ensure accurate tracking of biological material and associated data.

Together, these strategies empower biobanks to efficiently integrate, share, and analyze diverse datasets, regardless of their source or format.In anticipation of cross-border healthcare integration, we are aligning with the European Health Data Space (EHDS) and related frameworks to facilitate seamless data exchange and interoperability with EU Member States. The upcoming eHealth interoperability platform in Cyprus, led by Prof. Christos Schizas, director of the National eHealth Authority, aims to standardize health data formats, privacy protocols, and security measures, ensuring consistent and secure data flows across borders. This integration is expected to enhance patient mobility within the EU, enabling citizens to access their health records and receive informed care, regardless of location.

3. **Bioinformatics.** Bioinformatics plays a critical role in biobanking and genomics by enabling the efficient storage, analysis, and interpretation of large-scale biological data. It bridges the gap between raw genomic data and actionable insights, supporting research, clinical applications, and Biobank operations which include:

- Genomics data processing and analysis through essential tools and pipelines that process raw genomic data into usable formats for research and clinical purposes that usually break down into, sequencing data processing following quality control, alignment, and variant calling of high-throughput sequencing data (whole-genome or exome sequencing), then variant annotation which links genetic variants to known databases (ClinVar, dbSNP, etc.) for functional and clinical interpretation and further downstream with multi-omics integration which combines

genomics, transcriptomics, proteomics, and metabolomics data for comprehensive analyses.

- Data management and storage to handle the vast amounts of data generated by our Biobank requires advanced bioinformatics systems with centralized storage solutions for raw and processed genomic data, data compression with efficient algorithms to store large-scale sequencing datasets without loss of critical information, and scalable platforms to manage data storage and on-demand computational resources.
- Our Biobank rely on bioinformatics to identify and interpret genetic variations for population genomics with the identification of allele frequencies and population-specific variants for association studies, Pathogenicity Prediction: Using in silico tools (e.g., REVEL, AlphaMissense, ACMG) to assess the clinical significance of genetic variants, and pharmacogenomics by linking genetic variants to drug response and adverse reaction data.
- Data visualization and reporting of complex genomic data for interpretation such as genome browsers in our internal installation of OpenCravat, interactive dashboards through OpenCravat, OpenSpecimen or REDCap for researchers to explore biobank data interactively and in the diagnostics section, automated reporting with clinical-grade variant interpretation reports.
- Secure and privacy-preserving data analysis where bioinformaticians ensure secure handling of sensitive genomic data with data encryption at rest and during transmission, and federated analysis where specific systems allow data analysis without transferring raw data, enhancing privacy.
- Research and collaboration support, where the bioinformatics tools enhance the utility of Biobank data for collaborative research using data query portals that enables researchers to explore and access Biobank datasets for specific hypotheses, providing collaboration platforms to facilitating joint analyses across institutions using shared computational environments, and meta-analyses by aggregating and analyzing data from multiple biobanks to increase statistical power.

4. **Machine learning and Artificial Intelligence**. A key feature of our approaches is the incorporation of advanced artificial intelligence (AI) and machine learning algorithms for data analysis, predictive modeling, and personalized health insights, with the aid of Dr Heimo Müller and his team at the Medical University of Graz. These systems enable real-time processing and analysis of large volumes of data, transforming raw health data into actionable insights that benefit both individual citizens and public health efforts. We have used AI in several aspects of our activities that include variant calling using deeplearning, variant discovery where AI algorithms can identify rare genetic variants and their associations with diseases, biomarker discoveries for prognosis, diagnosis and therapy response prediction, for predictive modelling, such asPolygenic Risk Scores (PRS) where AI-driven models analyze genomic variants to predict an individual's risk for complex diseases, and disease progression models which forecast disease trajectories based on longitudinal genomic and phenotypic data.

5. **Biobank Information Technology,** where our core Biobanking data management activities are supported through the OpenSpecimen platform, an industry-leading and dedicated Biobank Information Management System (BIMS). OpenSpecimen is a highly adaptable, open-source solution designed to manage the full lifecycle of biospecimens, from collection and processing to long-term storage and usage. In addition to robust data and metadata management, OpenSpecimen integrates with other digital health systems, enabling automated data capture, traceability, and secure, role-based access to sensitive health data. This centralized system forms a secure foundation for our Biobank operations and is adaptable for future integration into a national or European eHealth network, thereby enhancing interoperability and scalability for large-scale biomedical research.
6. **Set the baseline of the Cyprus Human Genome (CYPROME),** through integrating data emerging from Whole Exome Sequencing of at least 5000 Cypriots and Whole Genome Sequencing of another 1000 Cypriots, until the end of 2026. This objective will complete the reference genome of Cypriots and canvass the architecture of the Cypriot genome, at a healthy and morbid level, enabling prevention measures. This effort offers immediate collective benefits, placing Cyprus prominently on the global human genome map. It will serve as a unique tool for intensifying research on many diseases, with researchers accessing data in pseudonimized and confidential formats. The data generated already from the whole exome sequencing of DNA from 1446 participants of the general population have been deposited in a very systematic manner in the OpenCravat database, with a plethora of useful features. This wealth of information can be immediately used to evaluate findings of genetic investigations, including diagnostics. The most important feature of each DNA variant identified in patients undergoing a genetic investigation, remains its frequency in the respective population, which still remains unavailable regarding Cypriots specifically. Geneticists are then required to make assumptions by seeking relevant information on international population genomic data bases, such as gnomAD. Responding to this unmet need, our Center will generate a unique and freely accessible database presenting variant frequencies throughout the Cypriot genome, thus propelling the potency of genetic diagnostics and triggering population-specific research projects. Finally, the capacity of biobank.cy to generate high-quality genetic data, has enabled Cyprus to participate as an equal partner and get a share of finances in prestigious projects such as the European Genomic Data Infrastructure (GDI) consortium and the Genome of Europe (GoE) project, which aims to congregate genomic sequences from 100,000 Europeans as a start.
7. **Creating an idea and technology incubator** for new researchers to develop and improve diagnostic methods, discover new biomarkers, and identify molecular targets to enhance disease prevention and treatment in a precision and personalized medicine framework. The group of the founder and director of the biobank.cy, Prof. C. Deltas, always hired young postdoctoral fellows who were given the opportunity, under secure funding, to develop innovative hypothesis-driven research projects, or more applied projects aimed at immediately serving the medical

community. The biobank.cy now employs nearly 50 people, about 20 of whom are PhD holders engaged in research development or research management. There are frequent discussions and brainstorming sessions in smaller groups with the goal to generate new ideas and identify the needs in a very unstable and tantalizing ecosystem where funding is never enough, and where even excellent ideas and projects are not funded due to inadequate funding. The structure of the Center of Excellence is such that it fosters collaborations and interdisciplinary fermentation of ideas, preparing young researchers for the open world.

8. **Establishing Cyprus as a regional hub for medical research** to promote collaborations with other centers in Cyprus and networks in European, Mediterranean, and Middle Eastern countries. By combining forces, this initiative aims to improve understanding of genetic diseases and stimulate new drug discoveries, attracting interest from research centers and pharmaceutical companies. To this end, the MediEuro Network was created, involving 11 teams from 11 countries in the Mediterranean and Middle East, while Regeneron, a pharmaceutical company in New York is already investing in our efforts, by offering us free whole exome sequencing of up to 5000 Cypriot volunteers, on a collaborative basis. The goal is to expand this network further.

8.5 The Role of Volunteers and the Medical Community

A cornerstone of the Biobank's efforts and the success of the *CY*-Biobank project is encouraging Cypriot citizens to altruistically participate in the Biobank by granting access to their medical records and donating small blood samples and urine. Volunteers can provide health-related information through questionnaires, blood tests, and blood samples for genetic material isolation and preservation (Fig. 8.2). The CNBC granted initial approval for volunteer participation in 2011 (File Number: CNBC/ΕΠ/2011/04) and additional approval specifically for the *CY*-Biobank project in 2020 (CNBC/ΕΠ/2020/04). Access to records and biological material for research purposes is subject to approval by the CNBC and the Centre's internal evaluation committee (https://biobank.cy/apply-for-access/).

In terms of engaging volunteers, the role of the medical community is essential. Without the involvement of healthcare professionals, the project cannot achieve its full potential. Cypriot doctors can participate in building the Biobank as a national research infrastructure in three main ways:

1. **Completing each patient's medical record** via the General Health System (GHS) portal. The GHS will optimally support this project once electronic health records become standard practice. With patient consent, encrypted data can be accessed by researchers for studies, ensuring confidentiality.
2. **Encouraging patient participation** in the Biobank. It would be beneficial and appreciated if doctors spend a few minutes explaining to their patients the value

of their altruistic participation, highlighting the benefits for themselves, their families, and the wider community. If patients agree, they can be referred to the Biobank, presently located at the premises of the UCY Medical School, for a meeting with a trained research nurse. They will be informed about the project, sign the approved consent form, and answer a questionnaire about their person.

3. **Providing access to valid personal information**, including demographic and health data, and information about medication they might be receiving. After that, they will donate blood and urine for the needs of the Biobank.
4. **More active involvement** if desired, by coordinating with biobank.cy staff to participate in setting and achieving research goals of particular interest. For the Centre, it would be especially gratifying if public and private doctors expressed their preferences and contributed ideas for studies using biobank.cy's infrastructure.

8.6 The Biobank

The Biobank enrols volunteers of the general population who are informed about it through several means, including the website, through which they can directly arrange an appointment (https://biobank.cy/book-your-appointment/), the social media, the press (television, radio, newspapers, leaflets), and lectures and other awareness events organized throughout the year by the Biobank personnel. For general population biobanking, interested participants can be healthy or suffer from any disease. The Biobank registers information with the use of a structured and comprehensive questionnaire, and clinical phenotyping, including medication if any is taken, most of the times self-reported. Volunteers of the general population are preferably Cypriot residents, aged ≥ 18 years old. Data are collected on-site using well established applications, such as REDCap® (Harris, Taylor et al. 2009, Harris, Taylor et al. 2019), and stored in OpenSpecimen® (https://www.openspecimen.org/), ensuring security and eliminating collection bias. Specifically, REDCap® (Research Electronic Data Capture) is a secure, web-based application designed for data collection and management in research studies. As previously mentioned, OpenSpecimen® is used as the Biobank's Information Management System, by organizing information related to the collection, processing, storage, and distribution of samples. Data and measurements collected are as follows: personal and family history as well as clinical (complete blood count, biochemical) and biometric (blood pressure, height, weight) (Table 8.2). To date, 1446 people aged ≥ 18 years with wide geographical distribution based on the origin of their parents, have undergone Whole Exome Sequencing and are under scrutiny at various levels. This genome data represent the CYPROME_v3, as the first detailed Cypriot genome. Genomic data of the CYPROME-v3 are accessible in OpenCravat, a versatile user-friendly tool which allows easy access to a plethora of information regarding every variant identified in these genomes.

The consent form used by biobank.cy has been approved by the CNBC and provides a broad consent, permitting the use of the data and samples within the scope and objectives of the *CY*-Biobank project in Cyprus and abroad, without the need to reconsent for every different use. Volunteers are enrolled and samples are collected at the Biobank premises at the UCY Medical School or our satellite premises at Kennedy Avenue in Nicosia, or anywhere around the island during specially organized excursions in communication with local authorities or corporate administrations. Samples received by the trained Biobank personnel are registered, coded, and processed under standard operating procedures which includes automation and approved protocols. At the end of the processing aliquots are stored in -80ºC freezers, as follows: one concentrated DNA aliquot and 3 diluted (50 ul of 50ng/ulit); 2 plasma aliquots; 4 serum aliquots; 5 urine aliquots (Fig. 8.3). Depending on separate disease-focused projects, other biospecimen may be collected and stored such as saliva.

Access to data and/or biospecimens by researchers is streamlined after completing and submitting online the Access Request Form, which is subsequently evaluated by an internal Evaluation Committee. The internal evaluation is not intended to be particularly scientific and critical, but it aims to secure that the data/material which is collected and archived with great expense and time is to be used prudently by experienced researchers. Of course, every research proposal that applies to use Biobank data/material must have a prior approval by the CNBC. Upon approval, a Data Transfer Agreement and a Material Transfer Agreement (DTA/MTA) is signed between the parties.

Table 8.2 A list of biochemical analysis and measurements performed on each sample collected from the Biobank participants. The results are archived in the participants' record and also returned to the volunteer free of charge.

Urine and blood tests			
1	Full blood count (hemoglobin, hematocrit, red and white blood cells, platelets, etc.)	7	Triglycerides
2	Total cholesterol	8	Glucose
3	Good cholesterol (HDL-cholesterol)	9	Glycosylated hemoglobin HbA1C
4	Bad cholesterol (LDL-cholesterol)	10	Creatinine
5	Risk ratio (LDL/HDL)	11	Uric acid
6	Risk ratio (total cholesterol/HDL)	12	Urine analysis (blood and protein in the urine, specific gravity, pH, glucose in the urine, etc.)
Biometrics/Limidometrics			
1	Blood pressure	7	Body weight
2	Body height	8	Body fat percentage
3	Body water percentage	9	Visceral fat rating
4	Basal metabolic rate (BMR)	10	Daily calorie intake (DCI)
5	Metabolic age calculation	11	Muscle mass
6	Bone mass	12	Body mass index (BMI)

It is worth emphasizing that the great usefulness of Biobanks lies both in their separate operation and in their cumulative power. It was proved that for the constructive statistical study of common multifactorial diseases, the participation of thousands of volunteers and the subsequent collection of data and samples is needed. Therefore, EVERY country, large or small, must have its Biobank. Science will advance in the application of precision medicine only by studying the genetic makeup of the entire population of planet Earth combined with a better understanding of the environment contribution to disease. The biobank.cy has ongoing collaborations and open communications with many stakeholders, including the Ministry of Health, the State Health Services Organization, the Federation of Cyprus Patients' Associations, the Pancyprian Medical Association, research bodies and institutes, in Cyprus and abroad.

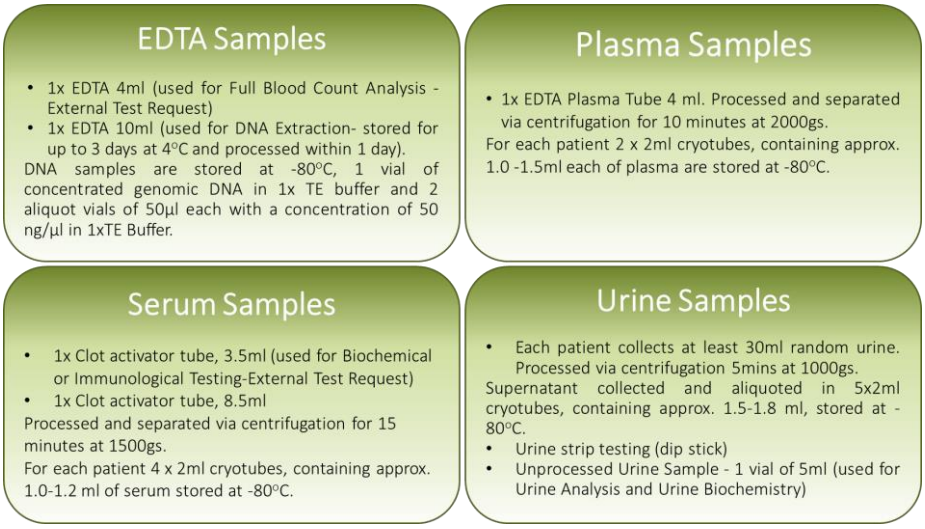

Fig. 8.3 Types of samples collected and processed under standardized procedures.

8.7 The CYPROME: Studying the Cypriot DNA

One of the main aims of our center has been the preparation of a reference Cypriot genome, aimed to canvassing the architecture of the DNA of Cypriots. To this end, we have already sequenced and pre-processed the genetic material of 1446 voluntarily enrolled individuals sourced from the general population using whole exome sequencing (WES). WES is a high-throughput technology which examines genome-wide protein-coding regions, using both wet and *in silico* laboratory methodologies. Although covering approximately 1% of the genome, these areas are the most probable of harboring pathogenic DNA changes. Examining that critical part of our genetic material WES is nowadays a cost-effective and fast approach to genetic research/diagnosis. It requires less computational resources for storage and

manipulation, while enabling simultaneous process of more samples compared to genome sequencing. On the other hand, WES is technically not capable of detecting certain genetic variations, such as large structural (copy number variations, CNV) and non-coding functional variants in deep intronic regions. For this, other supplementary laboratory techniques are often required for the detection of potentially elusive genetic changes in undiagnosed cases. The CYPROME is already in its third version, CYPROME_v3.

8.7.1 How CYPROME_v3 is being exploited

Exome sequencing of a population can be used in various applications that pertain to both research and diagnosis. Initially, knowing the genetic makeup of a population enables proper clinical evaluation of genetic variants of uncertain significance. This can reduce false positive findings benefiting the patient and the healthcare system. As far as genetically inherited phenotypes are concerned, WES can provide insightful clues about the health status of a population. By pinpointing the most frequent pathologies, healthcare system and research niche resources can be effectively and fruitfully managed. Moreover, by combining exome and sample origin data researchers can identify founder events that cluster in specific geographic locations. When communicated to clinical doctors, such information can be useful for early diagnosis of future cases. In addition, exome sequencing accompanied by demographic and clinical metadata can assist deciphering environmental influences on the population. Finally, exome sequencing can be utilized for the creation of a reference genome for the population of Cyprus. Currently, owing to political problems relating to the invasion and occupation of the northern part of Cyprus by Turkey, since 1974, we have not had access to the Turkish-Cypriot population which is mainly inhabiting the northern occupied part. The three minorities, Armenians, Maronites and Latins, are well represented in the Biobank and WES has been performed or is going to be performed soon.

8.7.2 OpenCravat database and its features

To unlock its full potential, exome sequencing needs to be easily accessible and browsable, as well as properly annotated. To meet such needs, we have taken advantage of OpenCravat, an open-source, swimmingly operable and scalable toolkit for genetic variant annotation and selection. Via our local installation we create custom variant annotations combining more than 300 annotation databases. Once commented, variants can be scrutinized via toolkit's graphical user interface in a statically and dynamically filterable tabular format. Advanced queries can also be built yielding highly specific results from complex and large input files. In total, OpenCravat offers effortless access to big genomic data interconnected with metadata collected from numerous databases and prediction tools.

8.7.3 Cyprome_v3 applications

Several significant packages of information are extracted from CYPROME examination. To begin with, our dataset is used to address various research questions, such as population structure, commonalities with geographically separated populations and levels of inbreeding. Understanding the underlying genetic architecture of the Cyprus population can have tremendous impact on clinical applications targeting specific parts of its general population. Exome data are also used to shape the site frequency spectrum for known deleterious variants in the population (see next paragraph) and compare it with that of other populations. As aforementioned, exome data are also utilized to obtain priceless insights for the prevalence of genetically inherited diseases in the Cypriot population. We envision that such pieces of evidence will be used for the evaluation and/or the modification of currently in-use healthcare directives.

8.7.4 Sequencing the general population is both useful and necessary in contemporary diagnostics

Differential diagnosis of complex and/or elusive pathologies has always been a crucial variable in selecting the most suitable treatment for suffering individuals, in the framework of precision medicine. More importantly, in the case of (suspected) genetically inherited deleterious conditions, such medical decisions usually affect both patients and their offspring, as well as other first degree or more distant relatives, many times spanning numerous generations. Nowadays, clinical genomics positively affect not only diagnosis, but also timely prognosis and preventive interventions for numerous genetically related conditions, including cardiomyopathies, nephropathies, eye diseases, and else. Although not directly related to the examined individuals, general population level high quality and high-throughput sequencing data can prove decisive in variant evaluation for the former. For instance, population allele frequencies can assist us in dissecting the importance of in other respects uncertain significance variants, thus addressing a gap in the predictive nature of variant classification tools. Furthermore, when combined to the patient’s clinical phenotype, population genomics can guide clinical doctors in critical decision making, including reference for a genetic test *per se*, thus enhancing the application of precision medicine.

8.8 Future Outlook

A future prospect that we hope will become a reality through the autonomy and strengthening of the country's hospitals, both in the public and private sector, is their connection to the Biobank's infrastructure, so that the archives will be available for

research in a coded fashion, provided that the volunteer's consent is first secured. In this way and if a scientific way of thinking is created in the public, we will also have in Cyprus every ***citizen-scientist*** who will be able to partake in the process and progress, with his/her active participation in research protocols that are in progress or that will be developed in the future. The connection of a nursing unit to a Biobank occurs in many hospitals in Europe, in a way that allows medical historical and imaging findings to be archived and biological material from thousands of patients to be stored daily, enriching the National Archives and contributing to research aimed to precision medicine. The maturation of the Biobank in a few years will provide in an easy and cheaper way material for research to the researcher of the next generation, in a way that was not possible until now, enhancing the prospect for new discoveries and innovative applications. It is emphasized that all procedures aim not to compromise the security and confidentiality of personal data, neither of the doctor nor the volunteer. This is ensured by the operation of a separate data quality and security assurance department and in communication with the UCY Data Protection Officer but also in cooperation with an external Data Protection Officer through a professional law firm, for the implementation of the General Data Protection Regulation.

8.9 Conclusions

The funding of the *CY*-Biobank project, following intense competition at the European level, is a major success for Cyprus's medical and scientific community. Established initially as a research infrastructure focused on the country's first Biobank, MMRC evolved into a Centre of Excellence, creating opportunities for next-generation biomedical research. The Centre provides access to high-quality records and biological materials from thousands of volunteers, serving Cypriot and, ultimately, global citizens. The unique characteristics of the Cypriot population's genetic pool, as documented in numerous publications over the last three decades, promise new insights that will enhance understanding, diagnosis, prognosis, and treatment of diseases.

The biobank.cy stakeholders underscore that investing in research and cultivating a research culture is a sound investment for improving quality of life and delivering personalized precision medicine while reducing healthcare costs over time. The robust funding of the new Centre of Excellence by the European Commission, the Republic of Cyprus, and the University of Cyprus guarantees the success of an ambitious health initiative involving many stakeholders. We conclude by affirming that every investment in the Biobank is an investment in a healthier Cyprus.

Acknowledgments
The *CY*-Biobank project is funded by the European Commission under the Horizon 2020 program, Grant Agreement No. 857122, and co-funded by the Republic of Cyprus and the University of Cyprus. Additional funding from various sources is supporting the several projects in progress under the premises of the biobank.cy Center of Excellence.

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Chapter 9
The Contribution of Biological Research to the Conservation of Flora of Cyprus

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"Study nature, love nature, stay close to nature.
It will never fail you."
Frank Lloyd Wright

Cite this chapter:

Georghiou, K., Stefi, A. L., Christodoulou, C. S., Andreou, M., Kounnamas, C., Paraskeva-Hadjichambi, D. and Kadis, C. (2025). The Contribution of Biological Research to the Conservation of Flora of Cyprus. In A. Ch. Hadjichambis, D. Mappouras, M. Hadjineophytou, P. Matsouka, P. Neophytou, M. A. Tsiarli, S. Constantinou (Eds). *Biological Cyprus: Trends and developments in biological research of the 21st century* (pp 237-274). Nicosia: Cyprus Biological Society. ISBN 978-9925-7814-3-0.

Cyprus Biological Society (CBS)

Chapter 9
The Contribution of Biological Research to the Conservation of Flora of Cyprus

Kyriacos Georghiou[1], Aikaterina L. Stefi[1], Charalambos S. Christodoulou[2], Marios Andreou[3], Constantinos Kounnamas[3], Demetra Paraskeva-Hadjichambi[4,5] and Costas Kadis[3]

1: Section of Botany, Department of Biology, National and Kapodistrian University of Athens, Panepistimiopolis, Athens 15784, Greece.
2: Ministry of Agriculture, Rural Development and Environment of Cyprus, Amfipoleos 6, Strovolos, 2025, Nicosia, Cyprus.
3: Nature Conservation Unit, Frederick University, 7th, Yianni Frederickou Street, Pallouriotissa, 1036, Nicosia, Cyprus.
4: Cyprus Center for Environmental Research and Education (CYCERE), Aghiou Andreou 306, P.O. 56091, 3304, Limassol, Cyprus.
5: Cyprus Ministry of Education, Sport and Youth, 1434 Nicosia, Cyprus.

Keywords: Seed Biology, Reproductive Biology, Plant Conservation, Seed Banking, Seed Germination, Conservation Biology

Chapter Abstract: A significant amount of research data concerning the conservation biology of the endemic, rare, and threatened plants of Cyprus has been accumulated during the past three decades. Most of this data was acquired through scientific work carried out by the Department of Botany of the National and Kapodistrian University of Athens (Greece) and the Nature Conservation Unit of Frederick University (Cyprus), in cooperation with the Department of Environment, the Department of Forests and the Agricultural Research Institute of the Ministry of Agriculture, Rural Development and Environment of Cyprus. These scientific projects focused mainly on seed biology and reproductive effort of the targeted plants and the acquired knowledge was utilized for in situ and ex situ conservation actions. Specifically, information on optimal germination conditions was used for ex-situ conservation of the targeted species in seedbanks, while a better understanding of the reproductive and germination mechanisms functioning in nature, contributed to in-situ species management. Another important outcome of this scientific research was the creation of databases for the documentation of seed banking and the conservation and protection status of the targeted species.

Acknowledgements
Special thanks are expressed to the Special Account for Research Grants of the National and Kapodistrian University of Athens, to the A.G. Leventis Foundation and to the Research and Innovation Foundation, since without their co-financing, the projects would not have been possible to implement.

9.1 Introduction

Since the end of the 1940s, the American philosopher and ecologist Aldo Leopold, in his famous quote "*We abuse land because we regard it as a commodity belonging to us. When we see land as a community to which we belong, we may begin to use it with love and respect*", aptly articulated the way many people treated the environment and the Earth.

Today, approximately seventy years later, both sustainable development and the protection of biodiversity are a necessary and sufficient condition for sustainability. Looking ahead to 2030, the United Nations (U.N.) includes among the Sustainable Development Goals (SDGs), the Goal 14 "Life on water" and Goal 15 "Life on land", which are directly related to the protection of biodiversity, while actions for the reduction of habitat loss, reforestation and the protection of ecosystems, are placed at the top of the priority list.

The current decade (2021-2030) has been designated as the "U.N. Decade for Ecosystem Restoration - UNDER" by the U.N. General Assembly (UN 2020; Abhilash 2021). The Convention on Biological Diversity (CBD) originally aimed to restore 15% of degraded ecosystems by 2020; the target that was not met and was renewed for 2030 with the updated goal of restoring 30% of degraded ecosystems (European Commission 2020; CBD 2020; CBD 2024a; Spiliopoulou et al 2023).

However, to reach the current level of recognition of the immediate necessity of protecting biodiversity, several years of intensive research and study, have passed; to achieve environmental awareness of the public and realization of the impact of the loss of natural ecosystems and the species living therein on human life, the environment and the economy, the strength has passed seems to be a difficult and long this road to "Ithaca". It is impressive that a very first important station on this "journey", a worldwide effort to raise awareness of all people around the world, was the designation of the year 2010 as the "*International Year of Biodiversity*" (Martens 2009; CBD 2010), while on 2000, the May 22nd will be celebrated every year as the "International Day for Biological Diversity (IDB)"; the moto for 2024 celebration is "*Be part of the Plan*" (UN 2000; CBD 2024b).

The year 1992 marks the starting point for the intensification of efforts to conserve global and European biodiversity (Montaz 1996), while in 1987 the concept of sustainable development was officially formalized when the entitled "*Our Common Future*" report, mainly known as Brundtland (named after Gro Harlem Brundtland who was the President of the Commission) was published (Brundtland-WECD 1987; Mezher and Park 2012). At the global level, the Earth Summit in Rio de Janeiro sees the signing of the Convention on Biological Diversity and at the European level the adoption of the Habitats Directive, which led to the creation of the Natura 2000 network (EC 1992; EC, 2009; Porteous 1996; Lawton 1997; Commission of the European Communities 2003), the largest network of protected areas in the world (European Environmental Agency), covering more than 18% of the European Union's land

area and around 6% of its marine areas (European Commission 2021; Gatti et al. 2023). The European Union has since adopted several policies and strategies to help protect biodiversity, recognising the threat that a possible loss poses to European ecosystems and the well-being of citizens. For example, **a)** European Biodiversity Strategy 2030, which was adopted in 2020 (EC 2020), is part of the European Green Deal (EC 2019) and sets targets to reverse biodiversity loss by 2030. It includes actions such as expanding protected areas, restoring damaged ecosystems and reducing pollution, while the estimated €20 billion annual budget for nature underlines that "nature" and conservation plans are set as a priority (EC 2020; Mammola 2020); **b)** the European Union financial instrument - L 'Instrument Financier pour l'Environnement (LIFE) - Nature and Biodiversity sub-programme- that supports projects for environmental protection and climate action and focus on the conservation of ecosystems, the protection of endangered species and the restoration of natural habitats (European Environment Agency); **c)** Common Agricultural Policy (CAP) introduced by 1962, now includes measures to protect biodiversity, such as agri-environmental payments, which reward farmers who adopt biodiversity-friendly practices, having in mind that the intensification of agriculture may contribute to the loss of biodiversity in Europe (Tilman et al. 2017; Hologšková et al. 2024). Under this prism CAP emerges as a tool to reverse this decline (Pe'er et al. 2020a).

Conservation Biology is a new, interdisciplinary branch of the biological sciences. The concept of Conservation Biology was introduced in 1968 by R.F. Dasmann (Dasmann 1968) as environmental conservation, while D.W. Ehrenfeld, two years later, introduced the term *Biological Conservation* (Ehrenfeld 1970). Conservation Biology "formed" in mid-80's, aims to investigate the human impact on biodiversity and to protect species with a limited geographical range and often high ecological or cultural value, through the development of practical tools and techniques to prevent species extinction (Georghiou, 1996; Heywood and Iriondo, 2003; Gerber 2020; Dobson et al. 2024). Prior to 1992, as early as 1989, Cyprus has been actively involved in this effort, by using national and European resources in the development of research programmes and in the implementation of *in situ* and *ex situ* conservation actions for species and habitat types. The *in situ* conservation actions were mainly implemented through LIFE research grants and concerned species and habitats of the Annexes to Directive 92/43/EEC.

Ex situ plant conservation, a strategy aimed at preserving biodiversity outside the natural ecosystems of plants, is used when plants face an immediate threat of extinction or when their preservation in their natural habitat is impossible due to habitat degradation (Engels and Ebert 2021). Owing to the numerous threats that biodiversity continues to face, the value of *ex situ* conservation is increasingly recognized. The most widely used methods of *ex situ* plant conservation include **a) Seed Banks:** Seed banks collect and store plant seeds under controlled conditions, where temperature and humidity are regulated to extend their lifespan. This is one of the most important and effective methods of plant conservation, as seeds can be stored for long-term use and reintroduced into the natural environment when conditions allow

(Wambugu et al. 2023). Furthermore, propagating plants from seeds enables the preservation of genetic diversity through recombination and contributes to the survival and natural selection of populations in vulnerable environments under pressure. For plants whose seeds cannot be stored in conventional seed banks, the cryopreservation technique (in liquid nitrogen at -196°C) is preferred (Davies et al. 2018; Ballesteros et al. 2021). Among its advantages is the fact that it is highly cost-effective and can represent a broad range of genetic diversity within a small storage sample. **b) Botanic Gardens:** Botanic gardens play a significant role in ex situ plant conservation by cultivating endangered plant species under controlled conditions, contributing to research on reproduction, genetic diversity, and the potential reintroduction of plants into their natural habitats (O'Donnell and Sharrock 2017). **c) Tissue Culture:** The tissue culture technique allows the propagation of plants from small tissue segments in sterile laboratory environments. This method is particularly useful for plants that produce few seeds or are difficult to store (Ruikar et al. 2024). **d) Gene Banks:** Gene banks preserve genetic material, including DNA, from seeds and shoots under freezing conditions (Rajasekharan 2015). Gene banks are vital for the conservation of genetic diversity and research into plant evolution and their capacity to adapt to new conditions.

The advantages of *ex situ* plant conservation include **a)** the preservation of species under immediate threat, **b)** the ability to maintain control, as germination occurs in laboratory-controlled conditions, **c)** research on seed development, by studying ecophysiological characteristics and the properties of each individual seed, **d)** genetic material is almost available for evaluation, and **e)** low cost and simple techniques (Maxted 2013). However, *ex situ* conservation, and specifically Seed Banks, is not a panacea! This is due to the difficulty in reintroducing seedlings into their natural ecosystem caused by challenges in adapting to the natural environment (Rais et al. 2024).

In the modern era, the significant augmentation and intensity of anthropogenic activities result in climate change (Prakash et al. 2022; Kabir et al. 2023). In the Mediterranean Basin, this crisis, now more intense than ever, is accompanied by rising temperatures, prolonged droughts, and reduced water resources, combined with extreme conditions, increased wildfires, soil erosion, and rising sea levels (Peñuelas et al. 2017; Dimitriadou and Nikolakopoulos 2021). It is impressive that the main causes of biodiversity loss implying anthropogenic actions, were first described in 1989 by the term "*Evil Quartet*" by J. Diamond and include habitat loss and fragmentation, overharvesting, introduced predators and competitors, and the indirect effects of these threats on ecological interactions (Diamond 1989).

Cyprus, as an island country in the eastern Mediterranean basin, lies at the heart of the impacts of the climate crisis. Human interventions, such as changes in the primary production sector, agricultural intensification and abandonment of farms, intensive tourism-related activities, deforestation, urbanisation, and intensive construction, exert severe and irreversible pressure on ecosystems (Bajoko et al. 2012; Fuladlu 2024), constantly necessitating measures and actions to protect its fauna and

flora.

The indigenous flora of Cyprus is divided into eight floristic regions (Meikle 1977; Delipetrou et al. 2008) and includes over 1,649 indigenous *taxa*, 276 adventive *taxa* occurring in the wild, 46 hybrids and 83 species with unclear status. Endemic species are represented by 141 *taxa*, while 33 species and subspecies are characterized as near endemic for which Cyprus bears significant responsibility for their conservation; the endemism rate is 8.55% of the indigenous flora (last update February 2023 - Hand et al. 2019).

For over 35 years, fieldwork and laboratory research methodologies have established a legacy and provide a guarantee for the continuation of efforts to protect the flora of Cyprus.

The outcomes of this research activity are reflected in a series of doctoral dissertations and undergraduate diploma theses, a significant number of presentations at local and international scientific conferences, publications in peer-reviewed international scientific journals and in research projects. *Ex situ* conservation actions resulted to the establishment of Seed Banks at (a) Laboratory of General Botany, National and Kapodistrian University of Athens (see § 9.3.1.), (b) Nature Conservation Unit (N.C.U.) Frederick University, (c) the Agricultural Research Institute (A.R.I.), and (d) the Department of Forests.

The establishment of Seed Banks in all these institutions enabled the simultaneous storage of seeds across the genetic banks, providing a safeguard against any potential issue that might suddenly arise in one of them. For the Laboratory of General Botany National and Kapodistrian University of Athens, the creation of a Seed Bank was the only appropriate and efficient method of contributing to the preservation and storage of seeds due to the distance from the habitats.

In general, *ex situ* plant conservation is a significant method for preserving biodiversity, particularly for endemic, threatened, and endangered plants. However, it should always be combined with *in situ* (within the natural environment) efforts to ensure that plants can survive and recover in the long term within their natural ecosystems.

Subsequently, in the following sections, the research projects conducted to protect the flora of Cyprus will be outlined, along with the scientific outcomes through these efforts; data is reflected in years of research (doctoral dissertations and theses), and have been disseminated to the scientific community through publications in peer-reviewed journals and conference presentations.

Throughout these efforts, bilateral collaborations between universities, research institutions, and the relevant authorities in Cyprus were essential for achieving integrated *ex situ* and *in situ* conservation actions.

9.2 Research projects related to Biological Research to the Conservation of Flora of Cyprus

The following research projects related to systematic research on the Conservation of Flora of Cyprus, can be classified into three categories:

A) Research projects aiming to documentation on the conservation status.

B) Research projects targeting on the conservation of endemic, rare and threatened plants of Cyprus.

C) Research projects focused on the exploitation of endemic plants of the flora of Cyprus.

9.2.1 "The Red Flora Data Book of the Flora of Cyprus"

- **Sponsored by:** Research and Innovation Foundation of Cyprus
- **Coordinated by**: Cyprus Forest Association
- **Partners:** National and Kapodistrian University of Athens; Environment Service; Department of Forests; Agricultural Research Institute
- **Duration:** 2002- 2006

The edition of a "Red Data Book" for the endemic, rare and threatened plants of Cyprus was a necessary and sufficient condition to document species within their habitats, aiming to implement measures for the conservation and protection of the flora of Cyprus. The species were mapped, their population sizes as well as the distribution ranges were assessed, their habitats were fully characterized and well documented and the threats and pressures they face within their habitats were also addressed. The conservation status of the species was evaluated according to the criteria of the International Union for Conservation of Nature (IUCN) and classified into categories, such as Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Regionally Extinct (RE), and others (Fig. 1a).

The data resulted from this research project is summarized below:

- 46 plants, including five endemic species, were classified as Critically Endangered.
- 64 species (nine endemic) were assessed as Endangered.
- 128 plants (31 endemic) as Vulnerable.
- 23 species were evaluated as Regionally Extinct none of these were endemic to Cyprus (fortunately!).
- Threats and pressures facing each species within their habitats were identified
- Protection and conservation actions per species were assessed.

It is worth highlighting that, to the best of our knowledge, this was the first time

a comprehensive and systematic evaluation of the plants' conservation status, conducted across the entire island, including the occupied territories.

- ✓ The Troodos National Forest Park was identified as the most significant area of endemism, sheltering 74 endemic *taxa*, while the whole Troodos Mountain range is home to 94 endemic *taxa*!
- ✓ The Paphos Forest, The Troodos National Forest Park, Madari region, the Akamas and Akrotiri Peninsulas, Cavo Greco, and the Pentadaktylos mountain range, emerged as the areas with the highest aggregation of threatened plants (which feature numerous narrow-endemic species or plants with restricted habitats.

This work was published in 2007, spanning 466 pages, by the Cyprus Forestry Association, in Greek with an English summary (Fig. 9.1b) (Tsintides et al. 2007). To conclude, this significant project showcasing the flora of Cyprus, contributed to identifying scientific gaps, opening new "avenues" for research, and providing in-depth information to both the Republic of Cyprus and international organizations responsible for biodiversity.

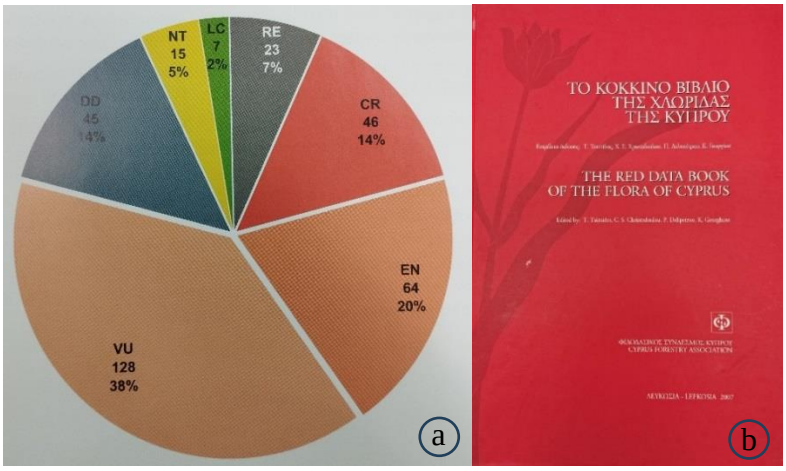

Fig. 9.1: a) Classification of the reviewed plants of the Flora of Cyprus into categories, according to the IUCN criteria; (CR) Critically Endangered; (EN) Endangered; (VU) Vulnerable; (RE) Regionally Extinct; NT (Near Threatened), DD (Data Deficient); LC (Least Concern). **b)** The cover page of the Red Data Book of the Flora of Cyprus.

Data from this project, was also communicated to an international conference (Georghiou et al. 2007).

9.3 Research projects targeting on the conservation of endemic, rare and threatened plants of Cyprus

9.3.1 Ecophysiological studies on seed germination of endemic, rare and threatened plants of Cyprus.

- **Sponsored by:** Special Account for Research Grants (SARG), National and Kapodistrian University of Athens
- **Coordinated by:** National and Kapodistrian University of Athens
- **Partners:** Department of Forests; Environment Service
- **Duration:** 1989 - 1993

This scientific research grant served as a starting point for the conservation of *endemic*, *rare* and *threatened* species of flora of Cyprus, via the creation of a database, the collection of seeds and the establishment of a Seed Bank.
The Final Outcomes of this research grant are summarised below:

- **Photographic Archive:**

Approximately 500 slides featuring *in situ* photographs of plant species from the flora of Cyprus were archived and made available.

- **Database Creation**:

A database was developed for the first time, documenting information on the size of natural populations and the characteristics of reproductive biology, including flowering, pollination, seed production and dispersal, seedling emergence and establishment in the field, and vegetative reproduction methods.

- **Seed bank establishment:**

A seed bank was created in the Laboratory of General Botany, Section of Botany, at the National and Kapodistrian University of Athens (NKUA), to ensure the long-term conservation and storage of seed samples from endemic, rare and threatened plants of Cyprus. NKUA Seed bank hosts 76 *taxa* and 205 seed lots from plants of Cyprus. However, the Nature Conservation Unit of Frederick University, the Agricultural Research Institute and the Department of Forests, also shelter many *taxa* of plants from Cyprus (Table 9.1).

Table 9.1: Number of cyprian taxa and number of seed lots in Seed Banks.

Organization	Number of *taxa*	Number of Seed lots
Agricultural Research Institute (ARI)	498	2014
Department of Forests	90	205
National and Kapodistrian University of Athens (NKUA)	75	218
Nature Conservation Unit, Frederick University	48	125

The creation of the Seed Bank in NKUA, one of the most powerful tools for ***ex situ* conservation**, was completed in 1991. It became the foundation for new research projects aimed at reintroducing plants into their natural habitats and collecting seeds from additional species of flora of Cyprus.

Furthermore, the availability of stored seeds, at any given time, facilitated the undertaking of undergraduate diploma theses and doctoral dissertations on the ecophysiology of seed germination of endemic, rare and threatened plants of flora of Cyprus.

The data derived from the research grant, is included in: Georghiou et al. 1991, Daskalakou et al 1991; Delipetrou and Georghiou 1991; Skordilis and Georghiou 1991; Kadis and Georghiou 1991; Georghiou et al. 1992; Kadis and Georghiou 1992; Tsabassi and Georghiou 1992, Daskalakou et al. 1991 and Daskalakou et al. 2015. Data on optimal conditions for the germination of seeds are also presented in Table 9.2.

9.3.2 Ecophysiological study on the germination of strictly protected plants of Cyprus.

- **Sponsored by:** Special Account for Research Grants (SARG), National and Kapodistrian University of Athens
- **Coordinated by:** National and Kapodistrian University of Athens
- **Partners:** Department of Forests; Environment Service
- **Duration:** 1993 - 1994

The objective of this research grant was to study the ecophysiology of seed germination of strictly protected plants of Cyprus's flora. Strictly protected plants are defined as species listed in Appendix I of the Bern Convention (79/90/92) on the Conservation of European Wildlife and Natural Habitats. For the great majority of these

species, the laboratory study of germination involved the investigation of the germination-rate dependency on temperature for each species, while identifying any dormancy, and determining methods (e.g. chemical scarification, gibberellic acid-GA_3, etc.) for breaking it. Furthermore, important parameters were also monitored; moisture content, seed dry weight, germination percentage and speed of germination. This ecophysiological study was conducted both on plant material previously collected (see § 9.3.1.) and on new collections. For most species, the optimal conditions for germination, as well as any specific treatments required, were identified and are presented in Table 9.2. Data was also communicated in Kadis and Georghiou 1993; Tsabassi et al. 1993; Thanos and Georghiou 1993; Delipetrou et al. 1993, Georghiou et al. 1993 and Kadis, 1994, Kadis and Georghiou 2010 and Kadis et al. 2010.

9.3.3 Conservation of endemic, rare and threatened plants of Cyprus.

- **Sponsored by:** A. G. Leventis Foundation
- **Coordinated by:** National and Kapodistrian University of Athens
- **Duration:** 1992 - 1995

Beyond the support provided by the National and Kapodistrian University of Athens (NKUA) for conservation initiatives in Cyprus (§9.3.1 and 9.3.2), the A.G. Leventis Foundation also supported, concurrently with the previously mentioned research project, a new one, providing three-year scholarships for the completion of doctoral dissertations by P. Delipetrou and C. Kadis (Kadis 1996).

Data from this research grant is presented in Kadis and Georghiou 1994; Hadjikyriakou et al. 1996; Paraskeva et al. 1996; Kadis and Georghiou 1997.

9.3.4 Conservation Biology of Four Plant Species of Priority under Directive 92/43/EEC

- **Sponsored by:** Research and Innovation Foundation of Cyprus
- **Coordinated by:** Frederick Institute of Technology
- **Partners:** National and Kapodistrian University of Athens; Department of Forests; Environment Service
- **Duration:** 2006-2009

This three-year research project, financially supported PhD dissertation of M. Andreou (Andreou 2010), aimed to provide information on the biology of four of Cyprus's rarest and most threatened species, namely *Arabis kennedyae*, *Chionodoxa lochiae*, *Pinguicula crystallina* subsp. *crystallina*, and *Scilla morrisii*. These species are included in the Red Data Book of Threatened Plants of the Flora of Cyprus and

are considered as priority species under Annex II of the European Directive 92/43/EEC. Findings derived from this grant are available in: Andreou et al. 2006, Andreou et al. 2007; Andreou and Georghiou 2008; Andreou et al.2011;2015 and in Table 9.2

We have to underline that the research project titled as "Conservation management in NATURA 2000 sites of Cyprus", which had been ongoing since 2004 (see § 6.2.1.2), had already provided information on the populations, locations, distribution ranges while the first monitoring plans for the plants of Cyprus (namely for *Arabis kennedyae*, *Chionodoxa lochiae*, *Pinguicula crystallina* subsp. *crystallina* and *Scilla morrisii*) were published in Delipetrou and Andreou 2006.

9.3.5 The plants of Cyprus: an intergraded program of students and teachers' education for the ecology and the environmental sensitization.

- **Sponsored by:** Research and Innovation Foundation of Cyprus
- **Coordinated by:** University of Cyprus
- **Partners:** National and Kapodistrian University of Athens
- **Duration:** 2006-2009

The study investigated the population viability of *Astragalus macrocarpus* subsp. *lefkarensis* and *Ophrys kotschyi* populations. Three populations were monitored in seven permanent plots for four consecutive years, recording traits, population dynamics, habitat, and anthropogenic impacts. Data were analyzed using RAMAS®-Metapop and stochastic models to determine finite rates of increase and critical stages in the life cycle. Management in RAMAS®-Metapop was simulated using artificial introduction scenarios to determine the optimum developmental stage for introduction and the minimum interval for population management action. The research demonstrated that seedling survival and transition to saplings and juveniles were low, but plant survival was high in large size scales.

The study highlights the importance of monitoring populations and implementing appropriate management strategies to protect these species. Data were combined with actions aimed to raise environmental awareness for young students. Data were communicated to public in Paraskeva-Hadjichambi et al. 2009; Paraskeva-Hadjichambi et al. 2010a and Paraskeva-Hadjichambi 2010b.

9.3.6 The impact of climate change on the local endemic plants of Troodos National Forest Park.

- **Sponsored by:** Research and Innovation Foundation of Cyprus
- **Coordinated by:** Frederick Research Center
- **Partners:** National and Kapodistrian University of Athens; Department of Forests
- **Duration:** 2009-2012

This project, which financially supported the PhD dissertation of C. Kounnamas (Kounnammas 2015) focuses on the reproductive biology of plants, specifically seed germination, which is vital for the conservation and management of plant biodiversity and provides critical data on conservation status. Seed germination is examined in the context of climate change, which is anticipated to significantly affect plant reproductive biology. Numerous studies highlight the impact of climate change on the biodiversity of plants in Europe and the Mediterranean. The innovative aspect of this project lies on its exploration of the effects of climate change on the ecophysiology of seed germination. The research included both fieldwork and laboratory work aimed at determining the reproductive potential, success, survival, and emerging behaviour of plant species, across varying altitudes. A total of eleven plant species were selected for study: *Acinos troodi* (Post), *Allium exaltatum* (Meikle), *Alyssum troodi* Boiss, *Astragalus echinus* DC var. *chionistrae* (H. Lindb.), *Crocus cyprius* Boiss. & Kotschy, *Crypsis hadjikyriakou* Raus & H. Scholz, *Cynoglossum troodi* (H. Lindb.), *Lactuca tetrantha* B. L. Burtt & P. H. Davis, *Onosma troodi* Kotschy, *Salvia willeana* (Holmboe) Hedge, and *Saponaria cypria* Boiss.

Research into the geographical distribution of populations revealed an expanded area of occurrence for 8 out of the 11 plant *taxa* studied. Laboratory work focused on investigating the temperature dependency of seed germination, identifying dormancy types, and determining methods for breaking dormancy. Seed germination behaviour was studied in temperature and light simulation chambers, under both current and projected conditions.

To study climatic conditions, climate data from four climate projection models were utilised, with the KNMI model identified as the most suitable for the study. The results concerning the ecophysiology of seed germination are summarised in Table 9.2, and described in Kounnammas et al. 2009, as well.

9.4 Exploitation of Endemic Plant Species of Flora of Cyprus in Commercial Floriculture

- **Sponsored by:** Research and Innovation Foundation of Cyprus
- **Coordinated by:** Agricultural Research Institute
- **Partners:** National and Kapodistrian University of Athens
- **Duration:** 2002-2005

Within the framework of applied Conservation Biology, this research grant dealt with the ultimate goal of commercially exploiting specific wild and endemic species.

The acquired knowledge on the ecophysiology of seed germination of endemic and wild species of the flora of Cyprus, is now gaining practical and commercial significance, responding to the global increasing interest in the commercial exploiting and valorisation of these plant species.

Under this perspective, focused on establishing a living bank of propagating material, leveraging and showcasing the genetic resources of the flora of Cyprus, and building a research collaboration network with the potential to extend the work to other endemic species, the research began with the study of six plant species. Specifically, the germination capacity and the potential cultivation for large-scale production, were examined for the species *Arabis purpurea*, *Centaurea acamantis*, *Euphorbia veneris*, *Onosma fruticosa*, *Origanum cordifolium* and *Ptilostemon chamaepeuce* var. *cyprius*.

One of the significant outcomes of this transfer of learning from laboratory to systematic cultivation, was the acquisition and dissemination of expertise for designing and implementing detailed cultivation programmes for these species, to interested stakeholders in Cyprus. The interesting findings of this research grant are summarised in Chimonidou et al. 2003; 2005; Vlahos et al. 2005, as well as in Table 9.2.

9.5 Undergraduate diploma theses

As previously mentioned, the research grants posed scientific questions, which were addressed initially and primarily through numerous dissertations. The mandatory preparation of a dissertation during the final two semesters of studies in the Department of Biology at the National and Kapodistrian University of Athens introduces students to early-stage research questions while teaching them certain techniques. Many of these students, building upon their dissertation work, sought to delve deeper and specialise further, eventually beginning the preparation of a doctoral thesis. However, some diploma theses, aimed to study seed viability and storage conditions in the Seed Bank, especially after a significant amount of time had passed since the seeds were stored (e.g., ten years). A characteristic example is the examination of seed germination for *Origanum cordifolium* in 1992 and 2002, as well as *Arabis*

purpurea in 1992 and 2003. Although these dissertations did not produce new findings on the germination conditions of a species, the information obtained helped optimise drying, storage, and thawing conditions (to assess viability and germination potential). Indeed, storage conditions improved over the years, while newer and more advanced infrastructure replaced older systems, supporting the more technologically robust preservation of seeds.

A significant number of plant species—twenty in total—were investigated in these dissertations. These include *Alyssum akamasicum*, *Alyssum chondrogynum*, *Alyssum troodi*, *Arabis kennedyae*, *Arabis purpurea*, *Bosea cypria*, *Centaurea akamantis*, *Cleome ornithopodioides*, *Dianthus cyprius*, *Erysimum kykkoticum*, *Jurinea cypria*, *Origanum cordifolium*, *Phlomis brevibracteata*, *Phlomis cypria*, *Phlomis lunariifolia*, *Ptilostemon chamaepeuce* var. *cyprius*, *Sedum cyprium*, *Sedum microstachyum*, *Silene fraudatrix*, and *Teucrium divaricatum* subsp. *canescens*. Notably, almost all these species are endemic to Cyprus, with the exception of *Cleome ornithopodioides* and *Jurinea cypria*, which are also found in parts of Turkey.

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 1)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
1	*Acinos troodi* subsp. *troodi*	(VU);D2	YES	Chamae-phyte	0.36	40	10, 15	dark	20	Kounnamas 2015	 G. N. Hadjikyriakou; © C. S. Chris-todoulou
2	*Allium exaltatum* (Meikle) Brullo, Pavone, Salmeri & Venora	(VU); D1, D2	YES	Geophyte	1.04	100	15	dark	5	Kounnamas 2015	 © C. S. Christodoulou
3	*Alyssum akamasicum* B.L. Burtt	(VU); D2	YES	Chamae-phyte / Hemicry-ptophyte	0.55	100	10, 15	dark	20	Kadis 1996	 © C. S. Christodoulou

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 2)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
4	*Alyssum troodi* Boiss.	N/A	YES	Chamae-phyte	1.61-2.87	80	5	dark	120	Kounnamas 2015	© C. S. Christodoulou
5	*Arabis kennedyae* Meikle	EN; B1ab(i,ii i,iv)+ 2ab(ii,iii,i v)	YES	Hemicry-ptophyte / Therophy-te	0.2 ± 0.00	<10	5-25	dark		Andreou et al. 2011	© M. Andreou
						98	15	single irradiation with red light	2.5	Andreou et al. 2011	
6	*Arabis purpurea*	N/A	YES	Chamae-phyte	0.09	100	15	dark+room cond (after 18d)	42	Chimonidou et al. 2005	© Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM); © C. S. Christodoulou
						100	20	+room cond (after 18d)	42		
						100	15	dR	8	Andreou et al. 2024	

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 3)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
7	*Arabis cypria* Holmboe	N/A	YES	Chamae-phyte	0.08 ± 0.001	60	15	dark		Andreou et al. 2024	© Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM); © G. N. Hadjikyriakou
						94	15	WL	8	Andreou et al. 2024	
						95	15	dR	9	Andreou et al. 2024	
						95	15	NO3-	10	Andreou et al. 2024	
8	*Astragalus echinus* var. *chionistrae* (H. Lindb.) Meikle	(VU); D2	Near Endemic	Chamae-phyte	3.58-3.67	80	N/A	Dark/light		Kounnamas 2015	© G. N. Hadjikyriakou; © C. S. Christodoulou
9	*Astragalus macrocarpus* DC. subsp. *lefkarensis* Agerer-Kirchof et Meikle	(VU); D2	YES	Hemicry-ptophyte	41.03	100	10-25	chipping	20	Kadis 1996	© Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM); © C. S. Christodoulou; © G. N. Hadjikyriakou

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 4)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
10	*Cedrus brevifolia* (Hook. f.) A. Henry	(VU); D2	YES	Phanero-phyte	53.00x10^3 ± 1.78	80	5	dark	70	Daskalakou et al 2015	© C. S. Christodoulou
						90	20	Cold strati-fication+ dark (8weeks)	7	Daskalakou et al 2015	
11	*Centaurea akamantis* T. Georgiadis & G. Hatzikyriakou	CR; B1ab(ii,iii,iv)+ 2ab(ii,iii,iv)	YES	Chamae-phyte	2.64	70	10, 15	Light/dark red	14	Kadis 1996; Chi-monidou et al. 2005	© C. S. Christodoulou; © G. N. Hadjikyriakou
12	*Chionodoxa lochiae* Meikle	(VU) D2	YES	Geophyte	6.3± 0.18 (2007) 4.6 ± 0.09 (2008)	80	10	dark	41	Andreou et al. 2015	© M. Andreou
						10	10	10 mM NO_3^-	45	Andreou et al. 2015	

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 5)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
13	*Crocus cyprius* Boiss. et Kotschy	(VU); D2	YES	Geophyte	5.62	70	10	Chipping +dark	N/A	Kadis 1996	© C. S. Christodoulou
						10	10	Chipping +dark	40	Kounnamas 2015	
14	*Crypsis hadjikyriakou* Raus & H. Scholz	EN; B1ab(ii,iii,iv)+2ab(ii,iii,iv)	YES	Therophyte	0.26	N/A				Kounnamas 2015	© C. S. Christodoulou
15	*Cynoglossum troodi* H. Lindberg	(VU); D2	YES	Hemicryptophyte	6.15-8.60	60	15	Cold stratification, dark	0	Kounnamas 2015	© C. S. Christodoulou; © G. N. Hadjikyriakou

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 6)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
16	*Crocus hartmannianus* Holmboe	(VU); D2	YES	Geophyte	5.40	60	15		60	Kadis 1996.	© C. S. Christodoulou
17	*Ferulago cypria* H. Wolff	N/A	YES	Hemicryptophyte	0.70	67	5		64	Kadis 1996	© C. S. Christodoulou; © G. N. Hadjikyriakou
						85	15	White light	24		
18	*Lactuca tetrantha* B. L. Burtt & P. H. Davis	(VU); D2	YES	Hemicryptophyte	1.08-5.39	10	10, 15	Light/ dark	3	Kounnamas 2015	© G. N. Hadjikyriakou; © C. S. Christodoulou

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 7)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
19	*Micromeria cyprica* Kotschy	Non	NO	Chamae-phyte	0.02 ± 0.001	62	15		10	Kadis et al. 2010	© C. S. Christodoulou; © G. N. Hadjikyriakou
20	*Nepeta troodi Holmboe*	N/A	YES	Hemicry-ptophyte	0.38 ± 0.005	50	5		70	Kadis et al. 2010	© G. N. Hadjikyriakou; © C. S. Chris-todoulou
						95	20	+ GA3 (500 ppm) + Intermit-tent red light (5min/30 min) 8 weeks cold stratifica-tion	13		

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 8)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
21	*Prilostemon chamaepeuce* var. *cyprius* (Greuter) Chrtek & B. Slavik	N/A	YES	Chamaephyte / Phanerophyte	10	80	20		22	Chimonidou et al. 2005	© Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM); © C. S. Christodoulou; © G. N. Hadjikyriakou
22	*Onosma troodi* Kotschy	VU; D2	YES	Hemicryptophyte	7.93	67	15	Chipping, scarification, removal of seed coat	6	Kadis 1996	© C. S. Christodoulou
						25-80	5	Dark	100	Kounnamas 2015	

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 9)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
23	*Ophrys kotschyi* H. Fleischm. et Soo	VU; C2a(i)	YES	Geophyte		90	20	Malmgren +coconut water solu-tion (1mL/L)		Koutsovoulou et al. 2013.	© C. S. Christodoulou; © G. N. Hadjikyriakou
24	*Origanum cordifo-lium* (Aucher-Eloy et Montbert ex Benth) Vogel	VU; D2	YES	Chamae-phyte / Phanero-phyte	0.22 ± 0.001	82 - 88	20	dark	16 - 21	Kadis1996; Chi-monidou et al. 2005; Kadis and Georghiou 2010	© C. S. Christodoulou
						82 - 88	10,15		6-10		

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 10)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (°C)	Conditions	Days for final germ/tion	Reference	Image
25	*Phlomis brevibracteata* Turrill	VU; C2a(i)	YES	Phanerophyte	5.58 ± 0.001	82	15	light/dark	12	Kadis and Georghiou 2010	© Botanic Garden and Botanical Museum Berlin-Dahlem (BGBM); © C. S. Christodoulou; © G. N. Hadjikyriakou
						88	1	far red/dark	12	Kadis1996; Kadis and Georghiou 2010	
26	*Phlomis cypria* subsp. *occidentalis* Post var. *occidentalis* Meikle	VU; B1ab(iii,iv)+2ab(iii,iv)	YES	Phanerophyte	6.14 ± 0.015	72	15	dark	12	Kadis and Georghiou 2010	© C. S. Christodoulou
27	*Pinguicula crystalina* Sm.	VU; D2	NO	Hemicryptophyte	0.03	90	15	White light	8	Kadis 1996	© C. S. Christodoulou
							20	Red light	5		

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 11)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
28	*Ranunculus kykkoensis* Meikle	VU; D2	NO	Hemicry-ptophyte	0.90	22	15	-	-	Kadis 1996	© C. S. Christodoulou
29	*Salvia veneris* Hedge	VU; D2	YES	Hemicry-ptophyte	1.69 ± 0.034	90	15	-	8	Kadis et al. 2010	© C. S. Christodoulou
30	*Salvia willeana (Holmboe) Hedge*	N/A	YES	Chamae-phyte	7.23 ± 0.104	90	5	-	50	Kadis et al. 2010	© C. S. Christodoulou; © G. N. Hadjikyriakou
						84		8 weeks cold strati-fication	4	Kadis et al. 2010	

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 12)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
31	*Saponaria cypria* Boiss.	N/A	YES	Hemicry-ptophyte	1.40-1.74	90	15	Light/ dark, scarifica-tion	15	Kounnamas 2015	© C. S. Christodoulou
						80-100	10	6 weeks cold strati-fication	7		
32	*Scilla morrisii* Mei-kle	EN; B1ab(i,ii i)+2ab(ii, iii)	YES	Geophyte	6.3± 0.08 (2007) 5.3± 0.08 (2008)	85	15	dark	43	Andreou et al. 2015	© M. Andreou
						100	10	10 mM NO_3^-	24	Andreou et al. 2015	
33	*Sideritis cypria* Post	EN; D1	YES	Chamae-phyte / Phanero-phyte	1.00 ± 0.009	98	10-15		9	Kadis et al. 2010	© Botanic Garden and Botanical Mu-seum Berlin-Dahlem (BGBM); © C. S. Christodoulou, © G. N. Hadjikyriakou

Table 9.2 Optimal seed germination conditions for endemic, rare and threatened plants of Cyprus. Additional treatment for dormancy breakage is highlighted. (Part 13)

N	Species	Red Data Book of Cyprus category	Ende-mism	Life-form	Mean mass (mg)	% max germ/tion	Temp/re (ºC)	Conditions	Days for final germ/tion	Reference	Image
34	*Teucrium divaricatum* subsp. *Canescens* (Čelak.) Holmboe	N/A	YES	Chamae-phyte	1.07 ± 0.067	22	15-20		18	Kadis et al. 2010	© C. S. Christodoulou; © G. N. Hadjikyriakou
						90	15	GA3 (500 ppm)	12	Kadis et al. 2010	
						95	15	8 weeks cold strati-fication	10	Kadis et al. 2010	
35	*Teucrium kyreniae* (P. H. Davis) Hadjik. & Hand	LC	YES	Chamae-phyte	1.07 ± 0.012	98	15		13	Kadis et al. 2010	© C. S. Christodoulou
						95	20		11	Kadis et al. 2010	

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Biological Cyprus: Trends and developments in biological research of the 21st century

In an era of rapid biological developments, this open access book presents trends and developments of contemporary biological research in the 21st century. Nowadays, the enormous development of biological research shows important applications in health, environment, economy and society in general. Biological research is vital for a modern society, as innovations resulting from this research can contribute to economic development in the bioeconomy sector and can also contribute to improving the health and citizens' quality of life. Biology is the science of our century and the main protagonist of the 4th industrial revolution whose threshold we have already crossed. Interconnectivity, intelligent automation, the internet of things and the internet of systems cannot be realized without the essential contribution of biology. We are already talking about the *in vivo* clinical application of the CRISPR molecular scissor, for the correction of genetic errors. New generation of "intelligent" and innovative drugs, as a result of biological research, have are already been circulating worldwide for the gene therapy of diseases. Neurological repair and genetic engineering are booming. We live in the century of biology, and personalized precision medicine, which is the future of medicine, cannot be achieved, without the essential contribution of biological research. Educational systems must embrace and adopt to these substantial developments of the science of biology.

ISBN 978-9925-7814-3-0