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Future of Exosome Bioinformatics

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On potential limitations of differential expression analysis with non-linear machine learning models

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Editorial

We are thrilled to present the latest issue of EMBnet. journal, volume 28, featuring a collection of articles that cover a broad range of topics in bioinformatics and computational biology, including data analysis, tool development, and multi-omics data integration.

One of the highlights of this issue is the paper on developing a computational pipeline for identifying new antibiotic compounds from soil microbiomes. This work highlights the potential of metagenomics and machine-learning approaches in drug discovery and development.

Another noteworthy contribution is the review on the use of machine learning in the prediction of protein-protein interactions, which summarises recent advances in this field and discusses the challenges and opportunities for future research.

Furthermore, we are happy to showcase a selection of articles highlighting the use of bioinformatics to analyse single-cell RNA sequencing data. These articles offer insights into the intricate cellular heterogeneity within complex tissues and diseases.

We sincerely appreciate all the authors, reviewers, and editorial team members who have contributed invaluable to this issue. Their unwavering dedication and hard work have been instrumental in ensuring the success of this volume. We sincerely hope our readers will find the articles on this issue informative and inspiring.

Lastly, we invite all bioinformatics and computational biology researchers to submit their valuable work to EMBnet.journal. We wholeheartedly welcome original research articles, reviews, and perspectives encompassing every field facet.

Join our esteemed community of researchers and showcase your contributions to bioinformatics and computational biology.

Thank you for your continued support of EMBnet. journal, and we look forward to bringing you more exciting research in the future.

Erik Bongcam-Rudloff
Editor-in-Chief
erik.bongcam@slu.se

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Future of Exosome Bioinformatics

Dimitrios P. Vlachakis[1,2,3]✉, Kalliopi Io Diakou[1], George P. Chrousos[1,2,3]

[1]Laboratory of Genetics, Department of Biotechnology, School of Applied Biology & Biotechnology, Agricultural University of Athens, Athens, Greece

[2]University Research Institute of Maternal and Child Health & Precision Medicine, and UNESCO Chair on Adolescent Health Care, National and Kapodistrian University of Athens, "Aghia Sophia" Children's Hospital, Athens, Greece

[3]Division of Endocrinology and Metabolism, Center of Clinical, Experimental Surgery and Translational Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece

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Exosomes are under intense study as a promising means for drug or biomarker discovery, primarily due to their implication in intercellular communication and the emergence of disease states and their potential as a cutting-edge, natural delivery system at a nanoscale level. The proteins and nucleic acid cargo of exosomes has been at the center of exosome bioinformatic analysis in the context of health and disease, towards the hunt for novel biomarkers and diagnostics. However, the exosomal lipid composition has been emerging as an interesting target of study as well. Exosomes derived from different sources exhibit enrichment of specific lipid classes and various lipid compositions under different physiological conditions. Therefore, there is a mounting need for exosome lipidomic studies to build the foundation for novel therapeutic studies that use exosomal components. Bioinformatic pipelines are under development to efficiently identify, quantify and elucidate the exosomal lipids and their roles in disease. Cutting-edge bioinformatic tools, such as LipidXplorer, LUX Score, and LipidHome allow the execution of essential analyses such as shotgun lipidomics, and the detection of systematic differences in lipid composition and metadata processing. In the case of pancreatic cancer, an admittedly prevalent and life-threatening disease, these tools have yielded novel exosome lipid biomarkers. Furthermore, bioinformatic platforms such as "Lipidomics Informatics for Life-Science" enable fast and integrated access to these pipelines in a user-friendly manner.

Modern bioinformatic methods facilitate the processing of exosome lipidomics data stemming from mass spectrometry. A set of bioinformatic tools, developed and provided by the LIPID MAPS consortium[1], allow the prediction of structural components from an input of mass spectrometry data. In addition to the chemical structures, information on the exosomal lipid ontology is readily available. Meta-analysis of the exosomal lipidomics data is also made possible through the platform, which filters the data for non-lipid artifacts and corrects potential errors in the processing of MS spectra before storing the datasets.

Proteins that are secreted by exosomes can have a multitude of effects on the development of pathological conditions. In contrast to eukaryotes, where a signal peptide marks proteins that are to be secreted to allow passage through the ER/Golgi-dependent pathway, the proteins to be secreted by exosomes lack such a signal peptide. Bioinformatic methods can thus bridge the gap toward the effective prediction of protein secretion mediated by exosomes. Intuitive algorithms, such as random forest, can be trained on amino acid sequences of proteins secreted and not secreted by exosomes as a relevant feature, approaching the prediction problem as a classification problem. ExoPred, which implements this method, has been developed to identify proteins secreted by exosomes in vertebrates, marking the potential of cutting-edge algorithmic approaches for predicting and annotating distinct, exosome-secreted proteins and opening new pathways for the subsequent study of their potential role in cell communication.

The study of exosomal miRNAs can lead to discovering valuable information concerning the target mRNAs for these miRNAs. Especially in the context of disease and its progression, the differential expression of exosomal miRNAs represents a source of valuable data that can be analysed with bioinformatic tools. With the increase in computational power, machine learning algorithms are gaining popularity in medicine and exosomal analysis. The strength and adaptability of these methods allow the extraction of relevant and helpful information from the vast array of available databases, which are becoming increasingly open to the public and contain large and diverse biomedical data. The

[1]https://lipidmaps.org/

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development of powerful algorithms, such as LASSO[2] enables the extraction of relevant features from a large number of unrelated features. Hence, this modern algorithm can help in the identification of key exosomal miRNAs and mRNAs involved in complex diseases. When combined with adequate cross-validation methods for the strict evaluation of features and parameters, LASSO – and similar feature extraction algorithms – can effectively generate novel information concerning the exosomal RNAs implicated in pathologies.

In cancer screening and precision nanotherapeutics, extracellular vehicles, such as exosomes, from patients are prime candidates for liquid biopsies. Machine intelligence-driven classification methods are rapidly emerging as a solid support system that, when paired with tried-and-true analytical methods, such as time-dependent spectroscopy, can aid the early and accurate detection of malignancies. An assortment of machine learning approaches such as multilayer perceptrons, support vector machines, and AdaBoost random forest classifiers[3], have been coupled with fluorescence correlation spectroscopy (FCS) conducted on samples of blood-derived vesicles from cancer patients, with the end goal being the accurate classification of tissue-specific extracellular vesicles. In parallel, convolutional neural networks (CNNs), quantum CNNs, and networks such as ResNet[4], show promise as supplementary validation tools when trained on the spectral images generated by the FCS. Along with continuously evolving AI algorithms and refined experimental techniques for exosome characterisation, we believe that the future of exosome bioinformatics is very prominent and its role in precision and personalized medicine will prove invaluable in the years to come.

[2]https://corporatefinanceinstitute.com/resources/knowledge/other/lasso/

[3]https://medium.com/machine-learning-101/https-medium-com-savanpatel-chapter-6-adaboost-classifier-b945f330af06

[4]https://www.mygreatlearning.com/blog/resnet/

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Technical Notes

qualign: solving sequence alignment based on quadratic unconstrained binary optimisation

Yuki Matsumoto✉, **Shota Nakamura**

Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
Competing interests: YM none; SN none

Abstract

Bioinformatics has, among others, the issue of solving complex computational problems with vast amounts of sequencing data. Recently, a new computing architecture, the annealing machine, has emerged that applies to actual problems and is available for practical use. This novel architecture can solve discrete optimisation problems by replacing algorithms designed under the von Neumann architecture. To perform computations on the annealing machine, quadratic unconstrained binary optimisation (QUBO) formulations should be constructed and optimised according to the application. In this study, we developed an algorithm under the annealing machine architecture to solve sequence alignment problems, a known fundamental process widely used in genetic analysis, such as mutation detection and genome assembly. We constructed a QUBO formulation based on dynamic programming to solve a pairwise sequence alignment and derived its general form. We compared with conventional methods to solve 40 bp of pairwise alignment problem. Our implementation, named qualign, solved sequence alignment problems with accuracy comparable to that of conventional methods. Although a small pairwise alignment was solved owing to the limited memory size of this method, this is the first step of the application of annealing machines. We showed that our QUBO formulation solved the sequencing alignment problem. In the future, increasing the memory size of annealing machine will allow annealing machines to impact a wide range of bioinformatics applications positively.
Availability: the source code of qualign is available at https://github.com/ymatsumoto/qualign

Introduction

In bioinformatics, there are often discrete optimisation problems belonging to the NP-Hard or NP-complete class, such as sequence alignment, genome sequence assembly, and structural estimation (Wang and Jiang, 1994; Medvedev *et al.*, 2007; Crescenzi *et al.*, 1998). Current techniques are effective in solving such small-scale problems of aligning short sequences, but not large ones requiring finding all overlaps among vast sequences obtained from a huge genome. Recently, the amount of sequencing data generated using actively developing next-generation sequencing technologies is growing faster than Moore's law, an exponential growth of a dense integrated circuit over time (Mardis, 2011).

Annealing machines have emerged as a new computing paradigm and have become readily available for practical use with quantum (Jünger *et al.*, 2021) and complementary metal-oxide-semiconductor (CMOS)-implemented hardware (Boixo *et al.*, 2014; Yoshimura *et al.*, 2020; Aramon *et al.*, 2019). In particular, quantum annealing machines are one step ahead of general-purpose quantum computers because of their computation using quantum effects; moreover, they are expected to solve discrete optimisation problems [6, 9] efficiently. The various algorithms designed for von Neumann architecture need to be theoretically converted into a quadratic unconstrained binary optimisation (QUBO) formulation because annealing machines require a QUBO model as an input for computation (Lucas, 2014).

Local sequence alignment is one of the most fundamental processes in bioinformatics (Smith and Waterman, 1981). Although the exact solution to this problem can be obtained using dynamic programming, this process belongs to the NP-Hard class (Wang and Jiang, 1994). In this study, we developed a novel sequence alignment algorithm using QUBO formulation based on dynamic programming, named qualign, derived from QUbo-based sequence ALIGNment, to solve a local sequence alignment using an annealing machine.

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Materials, Methodologies and Techniques

We constructed a QUBO formulation to solve sequence alignment problems using the annealing machine as equation (1) represented by a Hamiltonian form. This equation is composed of three major terms. The first term assesses whether the alignment is matched or not according to the scoring of the BLOSUM62 matrix constant (Eddy, 2004). The second term represents the matching character between two input sequences and limits the number of aligned characters to one at most. The last term limits the occurrence of base swaps before and after a base when it is aligned.

$$H = C_0 \sum_{\substack{0\le i<N \\ 0\le j<N}} A_{s_i^1 s_j^2} x_{i,j} + C_1 \left(\sum_{0\le j<N} \left(1 - \sum_{0\le i<N} x_{i,j} \right)^2 + \sum_{0\le i<N} \left(1 - \sum_{0\le j<N} x_{i,j} \right)^2 \right)$$
$$+ C_2 \sum_{\substack{0\le i<N \\ 0\le j<N}} x_{i,j} \left(\left(\sum_{\substack{0\le i<N \\ 0\le j<N}} x_{a,b} \right) - \left(\sum_{a\le i \wedge b\le j} x_{a,b} \right) - \left(\sum_{i\le a \wedge j\le b} x_{a,b} \right) + x_{i,j} \right) \quad (1)$$

Here, N is the number of characters in an input sequence. xi,j represents whether i- and j-th characters were aligned, and their values were allocated to each qubit as the Boolean values. C_0, C_1, and C_2 represent the weight coefficient of each term. s_i^1 and s_j^2 represent the pairwise sequence alignment of the inputs. A represents the alignment-score matrix that returns a matching score between s_i^1 and s_j^2.

To formulate the sum of the products, the two input sequences were initially taken into the computer memory and converted to a QUBO formulation using the pyQUBO package (Figure 1). To solve the sequence alignment problem using the QUBO model, we designed qualign to set each of the coefficient variables in equation (1) to C0=4, C1=8, and C2=8 as the default setting. The converted QUBO model was solved using an actual annealing machine or simulated annealing sampler, dwave-neal provided by D-wave, the vendor developing the quantum annealing machine. Our design currently supports three kinds of solvers, including the D-wave Quantum annealing machine, the Fujitsu Digital annealer, and the dwave-neal solver.

Results

We evaluated the alignment accuracy of the qualign against other conventional methods when asked to align pair-wised sequences selected from the benchmark alignment database, BAliBASE (Thompson *et al.*, 1999) and trimmed the sequence length to 40 bp. The resulting alignment score using the qualign was -17, which improved from -30 without alignment. The alignment scores of ClustalW (Thompson *et al.*, 1994) and MUSCLE (Edgar, 2004) were -17 and -140, respectively (see Supplementary data file). These results indicate that the accuracy of qualign was comparable to the conventional software tools.

We developed a novel local sequence alignment algorithm based on a QUBO model using an annealing machine. Our model could be generalised to a multiple-sequence alignment with an arbitrary number of sequences. The general form of equation (1) is represented by equation (2), where i and j represent a set of indices and an element of I, the set of all combinations of indices, and the function ei(k) returns the k-th element of i.

$$H = C_0 \sum_{i\in I} A_{S(i)} x_i + C_1 \sum_{i\in I} \sum_{0\le m<M} x_i \left(1 - \sum_{\{j\in I \mid 0\le i(m)<N\}} x_j \right)^2$$
$$+ C_2 \sum_{i\in I} x_i \left(x_i + \sum_{j\in I} x_j - \sum_{\{j\in I \mid \forall k\, e_i(k)\le e_j(k)\}} x_j - \sum_{\{j\in I \mid \forall k\, e_j(k)\le e_i(k)\}} x_j \right) \quad (2)$$

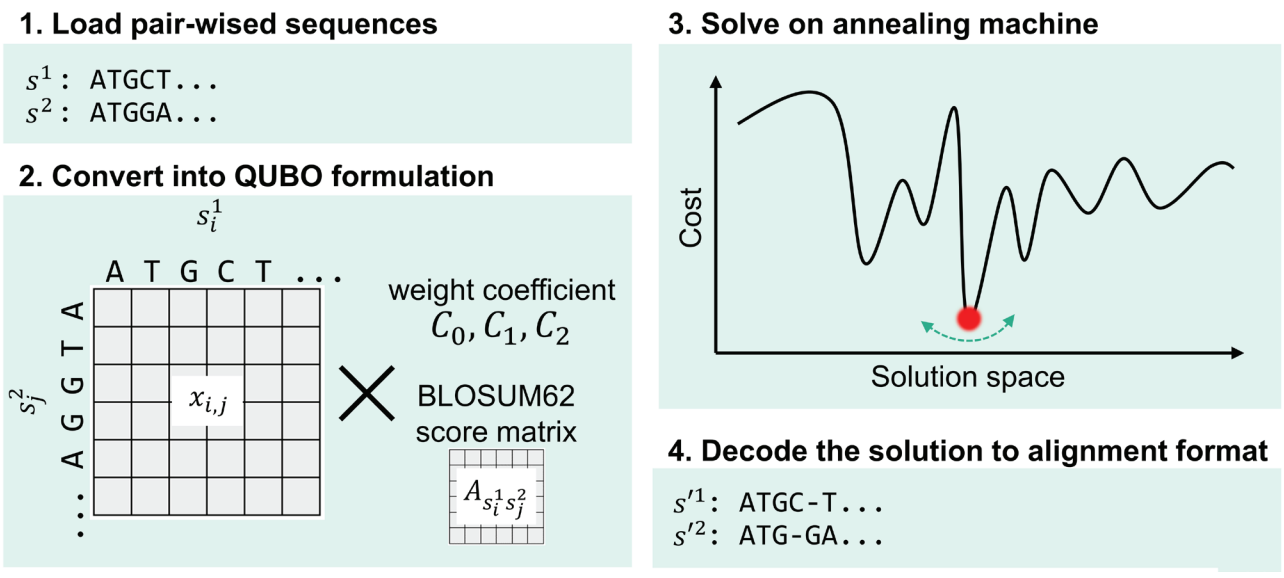

Figure 1. Algorithm workflow of qualign. The computation on the annealing machine is performed in step 3, steps 1, 2, and 4 are performed on a conventional computer.

Although the annealing machine could solve the problem in a given time, it required a QUBO model as the input. In the current implementation of qualign, the number of input sequences was limited to two due to restriction of the memory size of the annealing machines (Boixo *et al.*, 2014; Aramon *et al.*, 2019).

Conclusions

We showed that the proposed algorithm using QUBO model solved the sequence alignment problem using the new computational paradigm under annealing machines. The accurate sequence alignment could also lead to optimised results of genome assembly or mutation detection because sequence alignment was performed in the initial steps for these analyses. Therefore, increasing the memory size of the annealing machine in the future will positively impact a wide range of applications in bioinformatics.

Availability and requirements

Project home page: https://github.com/ymatsumoto/qualign
Operating system(s): Any platforms
Programming language: Python
Other requirements: dwave-neal, pyqubo
License: MIT Licence
Any restrictions to use by non-academics: No restrictions

Key Points

- A new computing architecture, the annealing machine, has emerged and is available for practical use.
- Computations on the annealing machine require constructing and optimizing quadratic unconstrained binary optimization (QUBO) formulations for the specific application.
- A QUBO formulation for solving sequence alignment was constructed based on dynamic programming, and our implementation, named qualign, demonstrated accuracy comparable to conventional methods.
- This is one of the first bioinformatics applications for annealing machines despite its current memory size limitations.

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Research Papers

A Bioinformatics pipeline for variant discovery from Targeted Next Generation Sequencing of the human mitochondrial genome

Lakshika Jayasekera, Kanchana Senanayake✉, Ruwandi Ranasinghe, Kamani Tennekoon

Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Sri Lanka

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Abstract

Sequence variants of human mitochondrial DNA (mt DNA) have been implicated in a variety of disorders and conditions. Massive parallel sequencing is becoming increasingly popular due to its efficiency and cost-effectiveness. In relation to acquiring significant sequence information like levels of heteroplasmy in mt DNA, it offers a marked improvement compared to previous methods used. Here we describe a variant calling pipeline for human mitochondrial DNA using Next Generation Sequencing (NGS) data obtained by enriching the sample only for mitochondria prior to sequencing.

Introduction

Human Mitochondrial (mt) genome is a double-stranded and a closed circular DNA molecule of 16,569 base pairs that represent <1% of total cellular DNA with each mitochondrion harboring 2-10 copies of mitochondrial DNA (mtDNA) molecules (Holt *et al.*, 2007; Veltri *et al.*, 1990). It codes for a total of 37 genes, including the 13 involved in electron transport and oxidative phosphorylation, 2 coding for 16rRNA and 12sRNA, and 22 coding for other mitochondrial transfer RNAs (tRNAs) needed for protein translation, thus proving its essential role in cellular function. (Anderson *et al.*, 1981). The presence of dissimilar sequences across different mitochondrial DNA molecules, from a single source, is referred to as heteroplasmy, which could conform to varying degrees among several tissues or different cells of the same tissue (Melton, 2004; Wong *et al.*, 2005). Compared to the nuclear genome, the mt genome has approximately 10 times higher mutation accumulation rate (Elmore, 2007), and it causes maternally inherited mitochondrial dysfunctions in a range of diverse disorders (mtDNA diseases) including diabetes mellitus, hypertension, Alzheimer's disease, heart diseases and cancer (Huang, 2011). There's also increasing evidence suggesting the association of somatic variants of mtDNA to other traits like ageing and cancer (Schon *et al.*, 2012). Thus, the characterisation of mitochondrial genome sequences is necessary for the molecular diagnosis of associated conditions. However, mtDNA analyses methods like PCR-restriction fragment length polymorphism (PCR-RFLP) analyses, Affymetrix's MitoChip, and even the gold standard Sanger sequencing fail to detect heteroplasmy under 10% (Mertens *et al.*, 2019). Further, these methods are hindered by the limited number of targets they can scan in a single run, highlighting the need for an accurate, cost-effective, and more sensitive method to study mtDNA.

Next Generation Sequencing and mtDNA analysis

Massive parallel sequencing has revolutionised the sequencing technology in recent years and proves ideal for small genome sequencing due to its high throughput and low cost (Yao *et al.*, 2019). Level of heteroplasmy detection could be significantly improved through these methods due to resulting high coverage and small size of the human mitochondrial genome. Although the different NGS technologies may use different methods in generating raw data, their final output is nucleotide base calls producing a huge number of 50 – 300 bp short reads (Mardis, 2013), usually combined in a FATSQ file.

Bioinformatics pipelines are an intrinsic aspect of NGS data analysis, to detect genomic alterations derived from these massive amounts of raw sequence data. The computationally intensive and complex nature of NGS data analysis makes many biologists who lack understanding of these computational techniques shy away from personally analysing raw sequence data (Roy

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et al., 2018). Several options are available to facilitate the analysis of mtDNA data acquired through NGS. However, their use is constrained by various factors. For example, several available bioinformatics pipeline frameworks like MitoSeek, Mtoolbox, are difficult to install, certain online servers like mit-o-matic, have limited input volumes or generate unreliable results (Weissensteiner *et al.*, 2016) In this paper, we present a bioinformatics pipeline for analyzing NGS data of targeted sequencing of the mitochondrial genome, through a series of command line tools.

Methodology

Quality assessment

When compared with Sanger sequencing, NGS acquire more errors as platforms face a variety of failures in chemistry and instrumentation, resulting in errors such as adaptor contamination, low-quality reads, and base call errors. To assure the conclusions derived through analysis are correct, it is necessary to eliminate these errors as downstream procedures fail to identify them (Pabinger *et al.*, 2014; Cox *et al.*, 2010; Dohm *et al.*, 2008). In this protocol, FastQC (version 0.11.8) (Andrews, 2010), the most preferred tool among the several tools available for checking the quality of raw data, was used for quality assessment. Upon assessment, this tool produces a report of useful information including quality score distribution across bases and across reads. Base quality score is an expression of base calling accuracy (Zhou and Rokas, 2014). A score of >20 is commonly referred to as the threshold for inclusion criteria of sequence reads, and removing sequences lower than 20 is preferable. As a part of the quality control procedure, adaptor sequences and the sequences not meeting the defined standards were removed using the cutadapt (version 1.18) tool (Martin, 2011). Similarly, sequences that were significantly shorter and longer than average were removed as well.

Alignment

Alignment is the process where the previously quality controlled massive amount of short reads, usually around 250 bp in FASTQ format, is mapped to the reference sequence that's in FASTA format, in this scenario, rCRS Human Mitochondrial Genome Reference sequence of Genbank accession No. NC_012920.1 (Andrews *et al.*,1999). It is the paramount step of any variant calling pipeline as even a few inaccurate alignments could produce many false-positive variant calls. For the current pipeline, the BWA-MEM tool of BWA aligner (version 0.7.12, one of the popular aligners that is fast and facilitates indel identification, -r1039) was used (Li, 2013). BWA was also used for the indexing of the reference sequence that was downloaded from Genbank prior to alignment, and the resulting sequence alignment mapping (SAM) format file from the alignment between the reference sequence and trimmed sequences was converted to a binary alignment map (BAM) file using SAMtools (version 1.9) BAM is the default binary format for storing sequence alignment data (Li *et al.*, 2009). Once the aligned BAM file is produced, alignment should be further refined. Accordingly, sorting and indexing of the BAM was also performed using SAMtools (version 1.9), through which BAM data is efficiently arranged and coordinate sorted, so the reads could be retrieved efficiently during further downstream analysis. SAMtools also compresses the aligned BAM file further before being used in deduplication (Li *et al.*, 2009).

Removing/marking duplicates

Duplicate removal is also enabled through SAMtools (version 1.9). This additional refinement process is important to mitigate the effects generated by the over-amplification of certain sequences. Duplicate sequences that are naturally present spanning the interested regions on DNA do not need to be removed. However, optical duplicates that are mistakenly read as separate clusters through signal capture software, when in reality they are generated by the same cluster, and the duplicates caused through PCR should be removed. PCR duplicates occur when the same amplified copies of one original fragment are identified as different fragments and further amplified through high throughput sequencing (Zhou and Rokas, 2014). In the initial steps of the NGS process, mtDNA is fragmented for library preparation and amplified for enrichment. Therefore, the presence of PCR duplicates at some level is usual. But having overly propagated duplicates cause erroneous end results. If a sequence subjected to PCR duplication contains a variant, that variant call would be biased. To further worsen the final output, if an error had occurred during PCR it would be further inflated during high throughput sequencing and cause a false positive. Therefore, compared with other DNA sequencing projects, the effect of duplicates is detrimental for heteroplasmy level detection of mtDNA analysis. Through deduplication algorithms, the groups of duplicate reads are identified and that of the highest sum of base quality scores is marked as a single read (Goto, 2011; Pfeifer, 2017).

Base quality score recalibration

The last stage that produces the final output of variant calling is possible through several different software tools. Just as many tools applied in different stages of the analysis, no single software could perfectly identify all variants in the genome of interest without false positives or negatives, however, according to a survey of variant analysis tools previously performed (Pabinger *et al.*, 2014), Genome Analysis Tool Kit (GATK) by Broad Institute of Harvard and MIT (McKenna *et al.*, 2010; Heldenbrand *et al.*, 2019) offers a satisfactory output for both germline and somatic variant calling. Prior to variant calling, Indel realignment and Base quality recalibration is the recommended practice. In previous versions of GATK, local alignment tools like IndelRealigner were available for Indel realignment.

However, in the current version, GATK 4 realignment is no longer recommended (Heldenbrand *et al.*, 2019). As per the GATK best practices pipeline (Van der Auwera *et al.*, 2013), before the input files are processed through GATK tools, they should be preprocessed with utility software. Accordingly, a sequence dictionary for the reference file is created using CreateSequenceDictioanry tool by Picard (version 2.25.1), whereas the read group information of the input BAM file is assigned using the AddOrReplaceReadGroups tool also by Picard (version 2.25.1), a step through which, all the reads of a single file are assigned into one read group. Sorting and indexing of this file, which is necessary for subsequent steps could also be integrated into the same command.

Owing to the systemic technical errors of NGS data processing, base quality score alone may not be a proper indication of true base call errors. To address this issue GATK has introduced base quality score recalibration (BQSR). According to the official website available at GATK[1], BQSR is the machine learning algorithm introduced by GATK that enables improved overall base qualities, that subsequently increases the variant call accuracy. With the latest version of GATK, 4.2.0.0, the process involves two major steps. Initially, a model of covariation along with a recalibration table is produced on the BAM input from the previous step, with the BaseRecalibrator tool, based on an indexed VCF file of known variants and SNP's downloaded from dbSNP for the respective reference sequence and various covariates including read group, machine cycle number, reported quality score, and nucleotide context. Secondly, using the ApplyBQSR tool, a new BAM output is produced with adjusted base quality scores, depending on the built model. Additionally, the effects of recalibration are assessed by building another model for the new BAM output with the BaseRecalibrator tool, and plots are generated with the AnalyzeCovariates tool to compare the effects of the process. It is noteworthy that the AnalyzeCovariates tool requires other tools installed like R libraries, ggplot2, gsalib, and reshape to function.

Variant calling and filtering

Variant calling of Human mitochondrial genome is possible through the mitochondrial mode of GATK (version 4.2.0.0) Mutect2 (Benjamin *et al.*, 2019). Through mitochondrial mode, it allows sensitive calling of short nucleotide variants and indels at high depths with local assembly of haplotypes. The output is a raw highly sensitive VCF call set. Due to various types of errors and biases in the data, it demand the generation of a high-quality set of variants. To identify false positives out of the original VCF files and acquire a balance between sensitivity and specificity, necessary filters should be applied through variant filtering. GATK (version 4.2.0.0) offers several filtering tools based on different strategies used (Pfifer, 2017; Van der Auwera *et al.*, 2013). FilterMutectCalls is the recommended tool for this scenario (Benjamin *et al.*, 2019) through which possible false-positive artifacts will be flagged in the output VCF by its 'failed filter' while the remaining would be marked as 'PASS' (Van der Auwera *et al.*, 2013; Roy *et al.* 2018). With the SelectVariants tool, they can then be excluded from the final VCF output file.

Generating coverage plots and VCF file report

With SAMtools (version 1.9), coverage plots can be created for the final BAM output of GATK (version 4.2.0.0). Additionally, with the DISCVRSeq (version1.21) tool, a VCF file report can be generated using a previously compressed and indexed final VCF file.

Determining heteroplasmy levels, contamination, and haplogroup detection

High throughput sequencing has enabled the detection of heteroplasmy levels that are below 10%, which was not possible with previous sequencing methods (Mertens *et al.*, 2019). mtDNA-Server (Weissensteiner *et al.*, 2016), a free online data analysis server for mtDNA, reliably detects levels of heteroplasmy in NGS data 1% or above. The standalone version of mtDNA-Server, which can be locally installed, Mutserve (version 2.0.0-rc7) was used to detect the level of heteroplasmy in this pipeline and provided a sorted and indexed BAM as input.

Sample cross-contamination during next generation sequencing of mtDNA proves to be challenging at the data analysis phase as they may present themselves as low-level heteroplasmy (Dickins *et al.*, 2014; Li *et al.*, 2010). Haplocheck is a software tool developed to estimate the contamination level of mtDNA samples through mitochondrial phylogeny (Weissensteiner *et al.*,2021). It can be used as a cloud service or be locally installed. Locally installed Haplocheck (version 1.3.2), was used in analyzing NGS data for contamination.

Distinct regions of the mtDNA genome sequence that group together, reflecting phylogenetic origin through different maternal lineages are defined as mitochondrial haplotypes. These haplotypes can be assigned to haplogroups that represent the main branching points of the mitochondrial phylogenetic tree as they show how specific SNPs or variations have been accumulated through a certain matrilineage (Pipek *et al.*, 2019; Samuels *et al.*, 2006). Determining haplogroup in mitochondrial DNA sequence studies is important both to trace these lineages in human population genetics and to identify their various associations with diseases and health conditions. Haplogrep 2 (Weissenteiner *et al.*, 2016) is a popular tool used to classify haplogroups in NGS studies. Local installation of Haplogrep 2 (version 2.1.25) was used to identify haplogroups of preferred VCF input files.

[1]https://gatk.broadinstitute.org/hc/en-us/articles/360035890531-Base-Quality-Score-

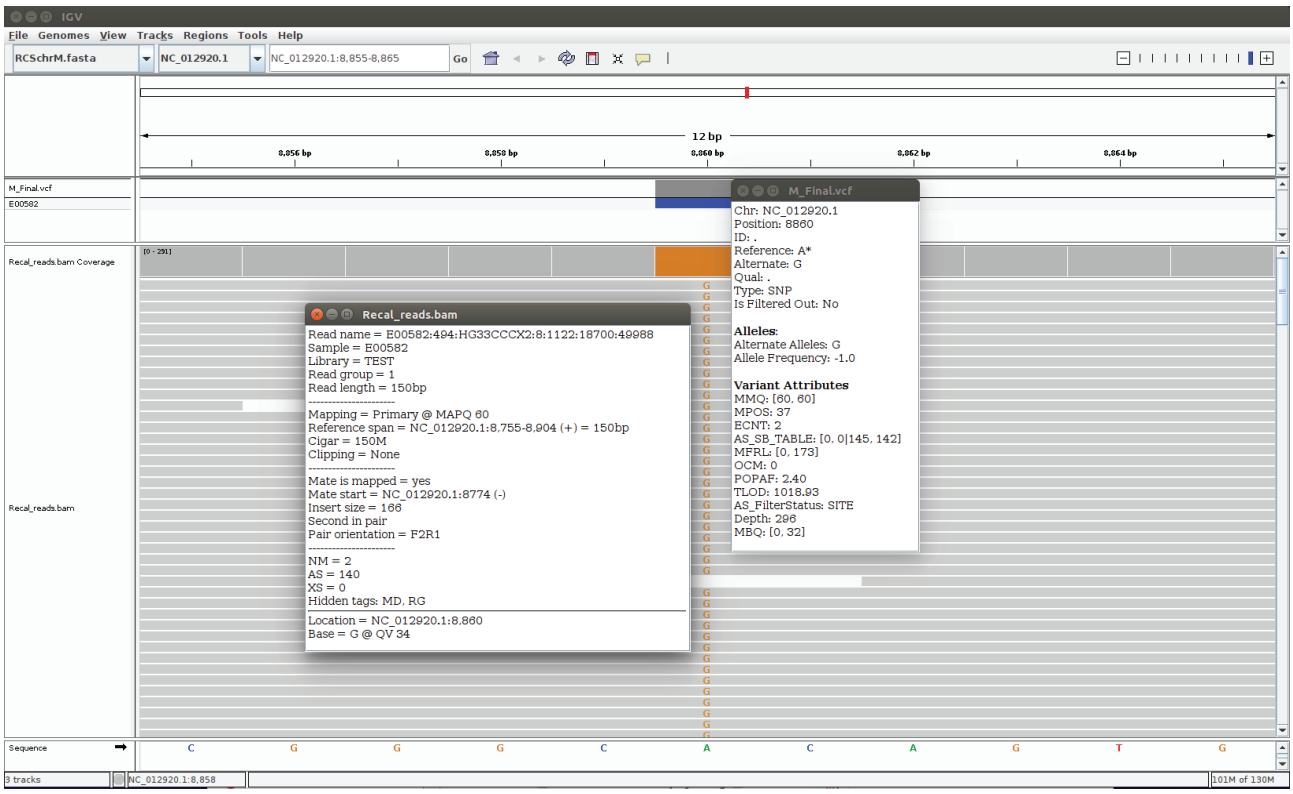

Figure 1. An A>G base substitution at position 8860 of human mitochondrial genome, aligned with rCRS Human Mitochondrial Genome Reference sequence zoomed for 12 base pairs length in IGV 2.9.2.

Results

Variant Annotation and visualisation

Assigning related biological information to identified variants is defined as variant annotation. There are several computational tools for human mitochondrial DNA analysis. Query with these tools provide sequencing data against variant databases and designate a set of associated metadata including the location compared to the reference sequence, respective change in amino acid and cDNA sequence, prediction of functional effects, and their presence in various databases (Roy *et al.*, 2018; Wadapurkar and Vyas, 2018). Variant Effect Predictor (VEP) (McLaren *et al.*, 2016) by ENSEMBLE project is a tool available at http://www.ensembl.org/ that accepts the final VCF file as input and enables the download of the annotated file in several formats. Via integrating SIFT (Kumar *et al.*, 2009) and Polyphen (Adzhubei *et al.*, 2010) it allows prediction of functional effect, as well as the discovery of genomic location, substitution effect of amino acid, and codon change. A txt format output, downloaded from VEP website, following annotation of the final VCF generated with the current pipeline is given in Supplementary file 5[2].

The final step of NGS variant calling pipelines is the visualisation of these data using genome browsers and visualisation tools. This task was performed with Integrative Genomics Viewer (IGV version 2.9.2) (Robinson *et al.*, 2011; Thorvaldsottir *et al.*, 2013) provided by Broad Institute of Harvard and MIT. It is a user-friendly and high performing interactive tool for exploring NGS data. By enabling read alignment examination, this step allowed further confirmation of called variant through visual estimation of it being true or a sequencing artifact. Additionally, through this step, more associated information of variants like mapping quality and variant impact acquired could be viewed individually (Figure 1).

Discussion

Limitations and perspective

Initially, it is important to notice that the sensitivity of heteroplasmy level detection correlates with increasing coverage depths (Holland *et al.*, 2011; Zhang *et al.*, 2012). At the same time, data generated with higher depths of coverage requires significant computer storage and advanced computers for data handling without compromising efficiency. Secondly, a signal from a very low-frequency variant is not discernable from that of a sequencing error. Finding the right balance between precision and sensitivity during data analysis, holds key to identify these variants. As desired, during variant filtering with FilterMutectCalls, the level for -f-score-beta argument could be adjusted between precision and recall, whereas 1 is the default value, 0.5 indicates higher precision over recall, and 2 indicates the higher recall over

[2]http://journal.embnet.org/index.php/embnetjournal/article/downloadSuppFile/1007/1007_supp_5

precision. However, other than bioinformatic analysis, criteria for quality control are also a limiting factor for detecting levels of heteroplasmies. It has been found that optimal conditions for primary PCR amplification and library preparation for high throughput sequencing are critically important as substandard conditions result in inflated variant frequencies (Mertens *et al.*,2019). Evidently, maximum accuracy in variant calling through NGS data demands high proficiency from laboratory practices to computational analysis. Nevertheless, despite the challenges present in the field of mitochondrial high throughput sequencing data processing, with rapidly improving and newly developing analysis tools coupled with other usual benefits offered via all massive parallel sequencing methods, NGS is likely to become predominant over previous sequencing methods in the foreseeable future.

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Key Points

- Targeted Sequencing of Human mitochondrial genome
- Bioinformatics pipeline for variant detection
- Next Generation Sequence data analysis of mtDNA

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Research Papers

Reliability and validity of the Dyadic Coping Inventory for Financial Stress in Greek couples

Joanne Maria Velegraki[1], Flora Bacopoulou[2], George P. Chrousos[3], Marilena Panagiotou[1], Orsalia Gerakini[1], Maria Charalampopoulou[1], Dimitrios Vlachakis[4], Christina Darviri[1] ✉

[1]Postgraduate Course of Stress Science and Health Promotion, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[2]Center for Adolescent Medicine and UNESCO Chair on Adolescent Health Care, First Department of Pediatrics, School of Medicine, National and Kapodistrian University of Athens, Aghia Sophia Children's Hospital, Athens, Greece

[3]University Research Institute of Maternal and Child Health and Precision Medicine, and UNESCO Chair on Adolescent Health Care, National and Kapodistrian University of Athens, Aghia Sophia Children's Hospital, Athens, Greece

[4]Laboratory of Genetics, Department of Biotechnology, School of Applied Biology & Biotechnology, Agricultural University of Athens, Athens, Greece

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Abstract

Financial stress can negatively affect a couple's relationship. The Dyadic Coping Inventory for Financial Stress (DCIFS) instrument assesses the way couples cope with financial stress. This study sought to validate the Dyadic Coping Inventory for Financial Stress (DCIFS) in Greek. The sample included 152 Greek couples (mean age: 42.82 ± 11.94). Confirmatory factor analyses provided support for delegated dyadic coping and evaluation of dyadic coping. Confirmatory Factor Analysis results supported a 33-item version consisting of the following subscales for both men and women: Stress Communication by Oneself and by Partner, Emotion and Problem-Focused Supportive Dyadic Coping (DC) by Oneself and by Partner, Negative DC by Oneself and by Partner, Emotion and Problem-Focused Common DC, and Evaluation of DC. The Dyadic Coping Inventory questionnaire and Perceived Stress Scale were used to assess the criterion validity of DCIFS.

Introduction

In times of economic turmoil, mental health issues such as anxiety and depression are reducing the well-being of the population (Viseu *et al.*, 2018). In recent years, due to the unstable economy, it is important to research the financial problems we face. Much research has been done on the problems couples face, but little has been said on financial problems (Falconier and Kuhn, 2019). The impact of chronic stressors, such as economic difficulties, is best understood within the context of one's close relationships (Karademas and Roussi, 2017). Significant stressors appear when there is an inability to meet economic needs. The couple that has economic hardships suffer both personally and as a couple (Kinnunen and Feldt, 2004). Stress influences communication, marital satisfaction, and the development of close relationships. Marriages subjected to chronic stress have a higher probability of ending up in divorce (Bodenmann *et al.*, 2006). There is increasing evidence that stress experienced by individuals in close relationships causes maladaptive relationship development, poor communication quality and decreased sexual functioning (Papp and Witt, 2010). Stressful experiences and financial stress can negatively affect a couple's relationship. Under financial stress, individuals tend to experience symptoms of depression, anxiety, or emotional distress (Falconier *et al.*, 2019). However, the negative behaviours that occur can be reduced if the couple is able to cope with stress together (Xu *et al.*, 2016). Dyadic coping is a stronger predictor of relationship satisfaction than individual coping (Herzberg, 2013). Taking into consideration that couples tend to be concerned about financial matters, it is obvious that financial stress is linked to negative effects within the relationship such as increased inter-partner hostility and aggression (Falconier *et al.*, 2019). Research on stress and coping in couples has received increasing empirical attention in North America and Western Europe. Generalisation of these findings may be limited due to the lack of variation in the contextual factors (such as culture and socioeconomic status) of the samples (Rusu, 2016). Since the early 1990s, authors

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emphasise the significance of the social context and the role of significant others in managing stressful encounters (Ledermann *et al.*, 2010). Evidence suggests that dyadic coping has a protective influence on marital quality, marital stability, and partners' well-being (Rusu *et al.*, 2016). Dyadic coping requires both partners mutually involved in the stress coping process such as providing and receiving support from each other and engaging in common problem-solving activities and shared emotion regulation (Traa *et al.*, 2014). It is important to understand how partners cope with financial stress. Among the many available models to understand couples coping with stress, also known as dyadic coping, the systemic-transactional model (STM; Bodenmann, 1995) offers the most comprehensive conceptualisation of dyadic coping, particularly when stressors can affect both partners. Bodenmann developed the Dyadic Coping Inventory (DCI), a self-report questionnaire specifically designed to measure dyadic coping (Gmelch , 2008). The DCI is the only available instrument that measures most aspects of the dyadic coping process, though it only addresses coping with stress in general, and does not address how couples cope with stress related to financial matters specifically. Given the need to understand couples' coping responses to financial stress and the potential benefits of an instrument that evaluates couples' dyadic coping with financial stress only, an adaptation of the DCI to assess couples' coping strategies regarding financial stressors has been created by Marianna K. Falconier (Falconier and Kuhn, 2019).

Materials, Methodologies and Techniques

Translation procedure

After receiving the authors' permission, the questionnaire was translated according to the World Health Organization's guidelines for the adaptation of instruments. A pre-test of the translated questionnaire was then held to identify the presence of unclear expressions. The participants of the pre-test were representative of the target population.

Participants and Procedure

The current study was conducted in Greece. The questionnaire was distributed to Greek couples by hand and online (google forms) from 2020 to July 2021.

Measures

Demographic data

The participants answered question regarding gender, date of birth, nationality, education level and job status, length of relationship, marital status, and number of children, as well as income satisfaction.

Dyadic Coping Inventory for Economic Stress

The DCIFS is a 33-item self-report inventory, designed to measure how couples cope with stress in general and not with a specific set of stressors. The DCIFS was adapted by the authors from the English version of the original 37-item DCI (Gmelch, 2008) to situations of financial stress. Similar to the DCI, items are rated on a five-point Likert scale (1 = very rarely to 5 = very often). Except for the Common DC and Evaluation of DC subscales (see Table 1 for specific items), the DCIFS includes the following subscales with a by Oneself item and a by Partner item: Stress Communication, Emotion-Focused Supportive DC, Problem-Focused Supportive DC–Negative DC, Emotion-Focused Common DC, Problem-Focused Common DC, and Evaluation of DC. The measure can yield a total score for DC resulting from addition of all item values after converting the Negative DC scores. The DCIFS can also produce two types of aggregated scales, DC by Oneself versus DC by Partner. A simple change of instructions to help participants respond in relation to financial stressors would not be sufficient to assess DC with financial stress due to the fact that some items are worded for stressors in general DCI items were adapted to make them specifically about situations of financial stress

Dyadic Coping Inventory

The DCI is a self-report questionnaire that was developed to measure all the dimensions proposed by STM. It initially included 55 five-point Likert-scale items (1 = very rarely, 5 = very often) (Bodenmann, 2006) but, as a result of factor analyses, the questionnaire was subsequently reduced to 41 items first and later on to 37 items (Bodenmann, 2008). The 37-item version of the DCI is widely used and validated in various languages assesses the various dimensions of dyadic coping with five different subscales: Stress Communication, Supportive DC, Delegated DC, Negative DC, and Common DC. Both Supportive DC and Common DC include two subscales: Emotion-Focused and Problem-Focused. Except for Common DC (Emotion-Focused and Problem-Focused), which assesses coping behaviours involving both partners, each of the other scales and subscales measure the respondent's perception of their own coping (by Oneself) and of their partner's coping (by Partner) in each of those dimensions. These dimensions result in the following 12 scales: (1) Stress Communication by Oneself; (2) Stress Communication by Partner, (3) Emotion-Focused Supportive DC by Oneself; (4) Emotion-Focused Supportive DC by Partner; (5) Problem-Focused Supportive DC by Oneself; (6) Problem-Focused Supportive DC by Partner; (7) Delegated DC by Oneself; (8) Delegated DC by Partner; (9) Negative DC by Oneself; (10) Negative DC by Partner; (11) Emotion-Focused Common DC and (12) Problem-Focused Common DC. The DCI includes a thirteenth scale made of two items to assess the respondent's

overall evaluation of DC. Scales can be aggregated in two different ways. On the one hand, subscales can be grouped into DC by Oneself and DC by Partner by adding all the scores from the by Oneself subscales and all the scores from the by Partner subscales respectively. On the other hand, subscales can also be grouped into Positive and Negative DC. Positive DC is the aggregation of the following By Oneself and By Partner subscales: Stress Communication, Emotion-Focused Supportive DC; Problem-Focused Supportive DC; Delegated DC; Emotion-Focused Common DC, and Problem-Focused Common DC. Negative DC is the aggregation of the subscales Negative DC by Oneself and by Partner. The DCI can also provide a total assessment for the couple's coping by adding the response values of each item after reversing the Negative DC responses. The DCI has been translated and validated in Greek.

Perceived Stress Scale

PSS is the most widely used psychological instrument for measuring the perception of stress. The PSS was developed to measure the degree to which situations in one's life are appraised as stressful (Cohen and Williamson, 1988). It has Likert-type scale with response categories ranging from 1 = Never to 5 = Very often (Taylor, 2015). PSS scores are obtained by reversing responses (*e.g.*, 0 = 4, 1 = 3, 2 = 2, 3 = 1 & 4 = 0) to the four positively stated items (items 4, 5, 7, & 8) and then summing across all scale items. A short 4 item scale can be made from questions 2, 4, 5 and 10 of the PSS 10 item scale. The PSS has been translated and validated in Greek.

Statistical analysis

Data are presented as frequencies N (%) for qualitative variables and as median and interquartile range (IQR) and means and standard deviations (SD) for quantitative variables. Confirmatory Factor Analysis (CFA) was conducted using SPSS Amos (Arbuckle, 2019). To confirm the hypothesised four-factorial structure of DCIFS (Stress Communication, Emotion-Focused Supportive DC, Problem-Focused Supportive DC, and Negative DC) by Oneself and by Partner for men and women separately. CFA was used to confirm the two-factor dimension (Emotion-Focused and Problem-Focused) of the Common DC for men and women separately. CFA was also used to confirm the 11-factorial structure of the total DCIFS (Stress Communication by Self and by Partner, Emotion-Focused Supportive DC by Self and by Partner, Problem-Focused Supportive DC by Self and by Partner, Negative DC by Self and by Partner, Emotion-Focused Common DC, Problem-Focused Common DC, and Evaluation of DC). Model fit was measured using the following fit indices: chi-square test ($\chi 2$), comparative fit index (CFI), the standardised root mean square residual (SRMR), and the root mean square residual of approximation (RMSEA). Considering that $\chi 2$ is sensitive to sample size, the recommended ratio of $\chi 2$/df to be smaller than 3 (Schermelleh*et al.*, 2003) was used to assess model fit. Good model fit is usually indicated by models reaching the following cut-off values (Hu and Bentler, 1999): CFI > 0.96, SRMR > 0.08, RMSEA < 0.06. However, models in which only the RMSEA index was slightly higher than 0.06 were not rejected given its likelihood of Type II error with small sample sizes (Chen, *et al.*, 2008). Normality of data distribution was tested and, as it was violated, non-parametric Spearman's rho coefficient was used to assess correlations. Correlations between DCIFS subscales were calculated in order to test overlapping between factors. A value >0.85 indicates a strong overlap. Also, correlations were calculated between DCIFS subscales and other measurements of the study. SPSS programme v.25 for Windows was used to perform statistical analyses and p = 0.05 was considered to be the level of significance for all analyses.

Results

Descriptives of the study's sample are presented in Table 1. Total participants' mean age in years was 42.82 ± 11.94, 44.16 ± 12.46 for men and 41.47 ± 11.28 for women. Participants reported being in a relationship with their partner for an average of 16.03 years ±12.52. Most of them were married; 167 ±70.8. Most married couples had 1-2 kids (204 ± 67.1). Concerning education background, most women had a Bachelor's degree (95 ± 62.5) while men had a lower percentage 78 ± 51.3. The most common job status was private employe; 100 ± 42.4. Most of the participants had a somewhat income satisfaction; 130 ± 55.1.

The initial four-factor model (Stress Communication, Emotion-Focused Supportive DC, Problem-Focused Supportive DC, and Negative DC) didn't show a good fit of the data for gender's reports in either the DC by Oneself or DC by Partner aggregated scales. (See Model 1 on Table 2).

The misfit of Model 1 could be attributed to low-factor loadings (<0.40). Chi-square is not good due to a large sample. Covariances were made between errors of the same group showing high M.I. The resulting second model fits the data significantly better than the first one for the aggregated scales. Men's reports of DC by Oneself: $\Delta\chi 2$ (55) = 130.28, p < 0.01; women's reports of DC by Oneself: $\Delta\chi 2$ (55) = 106.23, p < 0.001; men's reports of DC by Partner: $\Delta\chi 2$ (56) = 185.90, p < 0.001; and women's reports of DC by Partner: $\Delta\chi 2$ (57) = 190.09, p < .001. Despite the significant fit improvement over Model 1, Model 2 did not reach the values for fit indices in all the aggregated scales. Items 1/14 showed p > 0,01 in both gender's reports of DC by Oneself/Partner and were removed from the scale. The items 1/14 in the Stress Communication subscale refers to letting one's partner know that we appreciate his/her practical support, advice, or help on how to resolve the financial difficulties and is different from the other three items on the subscale about showing one's stress to the partner, telling the partner about one's stress, or asking partner

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Table 1. Participants' sociodemographic characteristics.

	Total	Male	Female	P-value
Gender N(%)				
- Male	152 (50)	-	-	
- Female	152 (50)			
Nationality N(%)				
- Greek	304 (100)	152 (100)	152 (100)	1
- Other	0 (0)	0 (0)	0 (0)	
Marital Status N (%)				
- Married	167 (70.8)	84 (71.2)	83 (70.3)	1
- Unmarried	69 (29.2)	34 (28.8)	35 (29.7)	
Age				
- Median (IQR)	41 (17.25)	42 (19)	40 (17)	0.081
- Mean (SD)	42.82 (11.94)	44.16 (12.46)	41.47 (11.28)	
Duration of relationship N(%)				
- Median (IQR)	12 (22)	12 (22)	12 (22)	0.973
- Mean (SD)	16.03 (12.52)	16.01 (12.55)	16.05 (12.54)	
Kids N(%)				
- None	2 (0.7)	1 (0.7)	1 (0.7)	1
- 1-2	204 (67.1)	103 (67.8)	101 (66.4)	
- 3+	98 (32.2)	48 (31.6)	50 (32.9)	
Education level N(%)				
- High school	56 (18.4)	33 (21.7)	23 (15.1)	0.128
- BSc	173 (56.9)	78 (51.3)	95 (62.5)	
- Msc/Phd	75 (24.7)	41 (27)	34 (22.4)	
Job status N(%)				
- Unemployed	17 (7.2)	2 (1.7)	15 (12.7)	
- Private employee	100 (42.4)	51 (43.2)	49 (41.5)	<0.0001
- State employee	39 (16.5)	15 (12.7)	24 (20.3)	
- Freelancer	80 (33.9)	50 (42.4)	30 (25.4)	
Income satisfaction N(%)				
- Not at all	26 (11)	12 (10.2)	14 (11.9)	
- little	31 (13.1)	14 (11.9)	17 (14.4)	0.682
- somewhat	130 (55.1)	63 (53.4)	67 (56.8)	
- A lot	47 (19.9)	28 (23.7)	19 (16.1)	
- Very much	2 (0.8)	1 (0.8)	1 (0.8)	
Income covers needs N(%)				
- yes	89 (37)	48 (40.7)	41 (34.7)	0.42
- no	147 (62.3)	70 (59.3)	77 (65.3)	
PSS Total				
- Median (IQR)	35 (14)	35 (14)	35 (15)	0.295
- Mean (SD)	35.82 (10.21)	35.07 (10.02)	36.58 (10.38)	
DCI Total				
- Median (IQR)	121 (22)	121.50 (22.75)	121 (22.25)	0.834
- Mean (SD)	119.70 (17.15)	119.74 (17.48)	119.66 (16.86)	

PSS: Perceived Stress Scale, DCI: Dyadic Coping Inventory

to do something. Showing appreciation may be different from communicating stress to partner and/or asking for assistance (Falconier *et al.*, 2019).

The Stress Communication subscale has offered challenges in previous validation studies. Model 3 was significantly better than Model 2. The third model indicated a good fit to the data for both men and women and for both by Oneself: men's reports: $\chi2$ (45) = 63.28, p = 0.00, CFI = 0.98, SRMR = 0.043, RMSEA = 0.05 (0.00–0.09); women's reports: $\chi2$ (45) = 46.65, p = 0.00, CFI = 0.99, SRMR = 0.034, RMSEA = 0.01 (0.00–0.12); and by Partner: men's reports: $\chi2$ (46) = 79.83, p = 0.00, CFI = 0.97, SRMR = 0.052, RMSEA = 0.07 (0.00–0.09); women's reports: $\chi2$ (47) = 102.194, p = 0.03, CFI = 0.96, SRMR = 0.051, RMSEA = 0.08 (0.02–0.12) aggregated scales. As Model 4 in Table 2 shows, fit indices for a two-factor model (Emotion and Problem-Focused Common DC) for both women's and men's reports showed a good fit of the model to the data: men's reports: $\chi2$ (4) = 11.98, p = 0.01, CFI = 0.99, SRMR = 0.012, RMSEA = 0.11 (0.00– 0.15); women's reports: $\chi2$ (4) = 5.235, p = 0.26, CFI = 0.99, SRMR= 0.009, RMSEA = 0.04 (0.04–0.20).

In the final analysis of the DCIFS factor structure, all the subscales were included simultaneously in an 11-factor model (see Table 2, Model 5). This model fit indices indicated an acceptable fit for men's reports, $\chi2$ (379) = 905.16, p = 0.00, CFI = 0.88, SRMR = 0.065, RMSEA = 0.09 (0.06–0.09), and a good fit for women's reports, $\chi2$ (379) = 788.85, p = 0.00, CFI = 0.91, SRMR = 0.056, RMSEA = 0.08 (0.03–0.07). A second model

Table 2. Confirmatory Factor Analysis.

		Men's reports						Women's reports					
		χ^2	df	p	CFI	SRMR	RMSEA	χ^2	df	p	CFI	SRMR	RMSEA
DC by Oneself and by Partner	Model 1												
	Oneself	212.389	59	0.000	0.869	0.1194	0.131	177.765	59	0.000	0.888	0.0924	0.115
	Partner	235.847	59	0.000	0.856	0.1323	0.141	198.545	59	0.000	0.906	0.1026	0.125
	Model 2												
	Oneself	130.286	55	0.000	0.936	0.1096	0.095	106.230	55	0.000	0.952	0.0884	0.079
	Partner	185.901	56	0.000	0.896	0.1215	0.124	190.090	57	0.000	0.915	0.0955	0.124
	Model 3												
	Oneself	63.285	45	0.000	0.983	0.0435	0.052	46.655	45	0.000	0.998	0.0346	0.016
	Partner	79.832	46	0.001	0.970	0.0524	0.070	102.194	47	0.000	0.960	0.0515	0.088
Common DC	Model 4	11.989	4	0.017	0.990	0.0122	0.115	5.235	4	0.264	0.999	0.0092	0.045
DCIFS Total	Model 5	905.167	379	0.000	0.883	0.0655	0.096	788.857	379	0.000	0.915	0.0561	0.085
	Model 6	824.530	375	0.000	0.900	0.0621	0.089	741.517	376	0.000	0.924	0.0537	0.080

Table 3. Descriptive characteristics of the subscales of DCIFS and total score.

Subscale	Items	Range	Mean	SD	Minimum	Maximum
FSCO	2,3,4	3-15	10.41	2.81	3.00	15.00
FSCP	15,16,17	3-15	10.06	2.97	3.00	15.00
FEFSDCO	18,19,22	3-15	11.46	2.16	3.00	15.00
FEFSDCP	5,6,9	3-15	10.81	2.50	3.00	15.00
FPFSDCO	21,25	2-10	7.01	1.54	2.00	10.00
FPFSDCP	8,12	2-10	6.43	1.75	2.00	10.00
FPFCDC	27,28,29	3-15	10.52	2.96	3.00	15.00
FEFCDC	30,31	2-10	6.99	2.12	2.00	10.00
FEDCO	32,33	2-10	7.20	2.07	2.00	10.00
FNDCO	20,23,24, 26	4-20	10.25	3.52	4.00	18.00
FNDCP	7,10,11,13	4-20	10.62	4.04	4.00	20.00
DCIFSTOTAL	All items	29-145	101.75	10.72	31.00	134.00

Table 4. Correlations (Spearman's rho) between DCIFS subscales and total DCIFS.

	FSCO	FSCP	FEFSD-CO	FEFSD-CP	FPFSD-CO	FPFSD-CP	FPF-CDC	FEF-CDC	FEDCO	FND-CO	FNDCP	Total DCIFS
FSCO	1											
FSCP	0.800**	1										
FEFSDCO	-0.484**	-0.466**	1									
FEFSDCP	-0.418**	-0.498**	0.686**	1								
FPFSDCO	-0.288**	-0.236**	0.692**	0.471**	1							
FPFSDCP	-0.306**	-0.364**	0.560**	0.738**	0.519**	1						
FPFCDC	-0.469**	-0.491**	0.735**	0.706**	0.604**	0.624**	1					
FEFCDC	-0.409**	-0.403**	0.617**	0.634**	0.559**	0.610**	0.772**	1				
FEDCO	-0.524**	-0.545**	0.676**	0.690**	0.509**	0.619**	0.810**	0.777**	1			
FNDCO	0.617**	0.644**	-0.569**	-0.553**	-0.394**	-0.492**	-0.577**	-0.525**	-0.597**	1		
FNDCP	0.679**	0.713**	-0.652**	-0.643**	-0.445**	-0.520**	-0.653**	-0.604**	-0.708**	0.785**	1	
Total DCIFS	0.367**	0.367**	0.296**	0.345**	0.416**	0.423**	0.368**	0.397**	0.271**	0.195**	0.132**	1

Note: *DCIFS: Dyadic Coping Inventory for Financial Stress*
***correlation is significant at the 0.01 level (2-tailed)*

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Table 5. Associations between DCIFS subscales and total score and other study variables.

Study measurements	FSCO	FSCP	FEFSD-CO	FEFSD-CP	FPFSD-CO	FPFSD-CP	FPF-CDC	FEF-CDC	FEDCO	FND-CO	FNDCP	Total DCIFS
DCI Total Spearman rho	-0.118*	-0.129*	0.341**	0.367**	0.398**	0.460**	0.461**	0.553**	0.447**	-0.234**	-0.258**	0.424**
PSS Total Spearman rho	0.571*	0.568*	-0.501*	-0.584*	-0.418*	-0.571*	-0.579*	-0.526*	-0.613*	-0.549*	0.660*	-0.06

Note: *PSS: Perceived Stress Scale*
*** Correlation is significant at the 0.01 level (2-tailed)*
** Correlation is significant at the 0.05 level (2-tailed)*

(Model 6 in Table 2) indicated a better fit. For men's reports: $\chi2$ (375) = 824.530, p = 0.00, CFI = 0.90, SRMR = 0.062, RMSEA = 0.08 (0.06–0.09), and a good fit for women's reports, $\chi2$ (376) = 741.517, p = 0.00, CFI = 0.92, SRMR = 0.053, RMSEA = 0.08 (0.03–0.07).

Descriptive characteristics of the subscales of DCIFS were calculated (See Table 3). The total score, including all items, was 101.75 (SD = 10.72), minimum and maximum range was 31.00 and 134.00, respectively.

Correlations (Spearman's rho) between DCIFS subscales and total DCIFS were calculated (See Table 4). There is a negative correlation between subscales because the results indicate values < 0.85. There is a strong positive linear relationship between Emotion-Focused DC by Oneself and Problem-Focused DC Common (FEDCO and FPFCDC: 0.81), as well as between Negative DC by Partner and Negative DC by Oneself (FNDCP and FNDCO: 0.78). There is a strong negative linear relationship between Negative DC by Partner and Emotion-Focused DC by Oneself (FNDCP and FEDCO: -0.70).

Associations between DCIFS subscales, total score and other study variables were calculated (See Table 5). DCI has a positive correlation with the DCIFS (DCI Total Spearman rho and Emotion-Focused DC Common/FEFCDC: 0.55). The PSS correlates positively only with 3 variables (FSCO: 0.57; FSCP: 0.568; FNDCP: 0,66). Total mean of DCI was 119.70; SD = 17.15 and PSS 35.82; SD = 10.21 (See Table 1).

Discussion

The goal of the present study was to validate DCIFS in a sample of Greek couples not seeking couple or family therapy. The results of CFA, led to the removal of two items from the Stress Communication subscale. The results supported a 33-item version consisting of the following subscales: Stress Communication by Oneself and by Partner, Emotion and Problem-Focused Supportive DC by Oneself and by Partner, Negative DC by Oneself and by Partner, Emotion and Problem-Focused Common DC, and Evaluation of DC. Confirmatory factor analyses showed that delegated dyadic coping by oneself and the partner and evaluation of dyadic coping were reasonable and reliable in terms of model fit and factor loadings.

Except for the Stress Communication subscale that was positively related to the Negative DC subscales, most subscales have a negative linear correlation meaning that most couples have a poor relationship. The fact that Stress Communication subscale was associated positively with Negative DC, could be suggesting that when couples cope with financial stressors, communicating stress by requesting support with finances, or showing financial stress through behaviour, may increase the likelihood of Negative DC through mutual blaming and may decrease the likelihood of providing Emotion-Focused Supportive DC and engaging in Common DC. Finances are what couples tend to argue the most. The results may be indicating that stress communication may not be as positive as perceived for some types of stressors. So communicating stress about finances through behaviour or by asking for financial support might not be beneficial for the couple's relationship (Falkonier *et al.*, 2019). Associations between DCIFS and Dyadic Coping Inventory (DCI) subscales have a positive correlation. The associations between DCIFS subscales show a negative correlation with Perceived Stress Scale (PSS) meaning that PSS is not the best tool to use along the DCIFS.

Conclusions

Although couples were mailed the questionnaires and were instructed to complete the measures independently, we cannot exclude the possibility that one partner completed both sets of questionnaires. Same with the internet version (google forms). The DCIFS has not been translated and validated in other languages apart from the original (English) so there is a restriction in comparisons with other countries. The model should be tested further. Future studies should seek to examine the broader applicability of this model to other dyads, including same-sex couples, which would provide evidence for its validity in a wider range of couples.

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Key Points

- Stressful experiences and financial stress can negatively affect a couple's relationship.
- Dyadic Coping Inventory for Financial Stress (DCIFS) is a self-report inventory, designed to measure how couples cope with financial stress.
- Validation of the DCIFS was performed in Greek couples.

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On potential limitations of differential expression analysis with non-linear machine learning models

Gianmarco Sabbatini, Lorenzo Manganaro✉

aizoOn Technology Consulting, Torino, Italy
Competing interests: GS none; LM none

Abstract

Recently, there has been a growing interest in bioinformatics toward the adoption of increasingly complex machine learning models for the analysis of next-generation sequencing data with the goal of disease subtyping (*i.e.*, patient stratification based on molecular features) or risk-based classification for specific endpoints, such as survival. With gene-expression data, a common approach consists in characterising the emerging groups by exploiting a differential expression analysis, which selects relevant gene sets coupled with pathway enrichment analysis, providing an insight into the underlying biological processes. However, when non-linear machine learning models are involved, differential expression analysis could be limiting since patient groupings identified by the model could be based on a set of genes that are hidden to differential expression due to its linear nature, affecting subsequent biological characterisation and validation. The aim of this study is to provide a proof-of-concept example demonstrating such a limitation. Moreover, we suggest that this issue could be overcome by the adoption of the innovative paradigm of eXplainable Artificial Intelligence, which consists in building an additional explainer to get a trustworthy interpretation of the model outputs and building a reliable set of genes characterising each group, preserving also non-linear relations, to be used for downstream analysis and validation.

Introduction

In recent years, high-throughput technologies for molecular data, such as next-generation sequencing (NGS) are getting increasingly cheaper (van Nimwegen *et al.*, 2016) and their use to improve our understanding of complex pathologies, such as cancer, is becoming widespread. This gives rise to an incredible amount of multi-omics data (genomics, transcriptomics, epigenomics, etc.), which can be analysed and exploited in the context of personalised medicine.

One of the main goals of these analyses is disease subtyping, meaning identifying a molecular-based stratification of patients affected by the same pathology, which ideally relates to prognosis. To this end, there is a growing interest in the adoption of state-of-the-art machine learning (ML) algorithms and models. These already proven excellent performances in almost any other field of application due to their ability to catch highly non-linear relations and patterns emerging from the dataset, which seems ideal when studying the biology of such a complex system as cancer (Zhang *et al.*, 2019; Tang *et al.*, 2019).

A very common approach to gene expression-based disease subtyping (see, *e.g.*, Su *et al.*, 2014) is to use a clustering model (an unsupervised ML model) based on molecular data to divide patients into groups, and then validating such grouping from a biological perspective by means of the so-called "downstream analysis". This latter typically consists in performing a differential expression (DE) analysis (Costa *et al.*, 2017; Soneson and Delorenzi, 2013) between the emerging groups to identify a set of genes which are considered determinants to discriminate between subtypes, and then exploiting those genes to perform a gene-set enrichment analysis (GSEA), unveiling the underlying biological processes characterising the emerging subtypes, and laboratory validation, whenever possible. Such an approach is of course valid, especially if a simple and linear clustering model has been used. However, when complex non-linear ML models are involved, DE-based characterisation has potential limitations that may affect the biological interpretability of results and that, to the best of our knowledge, has not been reported so far. In particular, when patient grouping is based on a non-linear relationship with a feature (*i.e.*, gene), such feature may be hidden to DE analysis, leading to a failure of the subsequent downstream analysis and validation and, overall, an incomplete biological characterisation.

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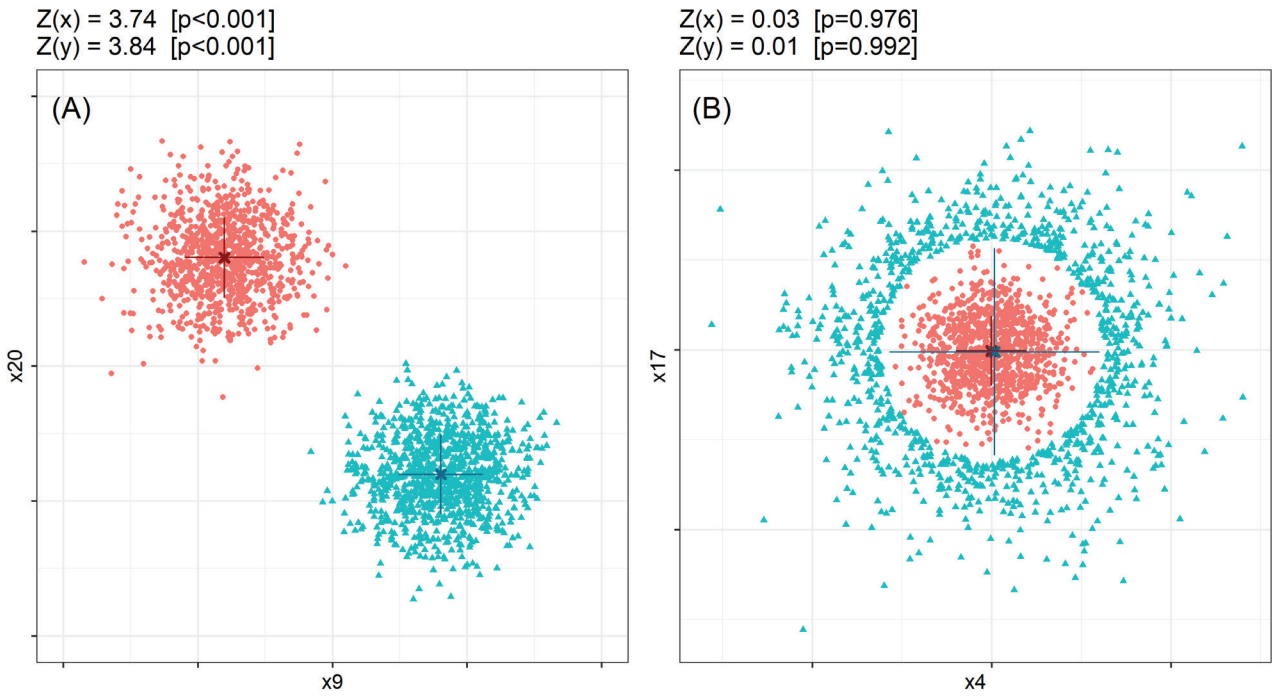

Figure 1. 2D representation of the synthetic datasets A (left) and B (right), where x and y axes represent the 2 relevant variables of each dataset respectively, namely: (A) clouds: x9, x20; (B) circles: x4, x17. Pattern of dataset (C) is not reported to avoid redundancy (similar to panel B). Colours and point shapes help in visualising the 2 emerging groups of each dataset. Thick crosses with error bars represent the mean and standard deviation of the 2 groups. Z(x) and Z(y) in the panel headers report the results of Normal Z tests between the mean values along x and y axes of each plot, respectively, with associated p-values.

Beyond unsupervised disease subtyping, such limitation of DE analysis in general still holds for any problem involving biological characterisation of different groups identified by exploiting non-linear ML models, such as the classification of high/low-risk groups for a specific end-point based on gene expression data (see, *e.g.*, Choi *et al.*, 2020).

In the subset of cases where the model developed provides the possibility to assign labels to new data points (*e.g.*, any supervised classifier or any unsupervised model that creates a partition of the feature space, such as kMeans-based models), we encourage the adoption of the innovative eXplainable Artificial Intelligence (XAI) paradigm (Arrieta *et al.*, 2020). It consists in building an additional explainer model to get a trustworthy interpretation of the model outputs as an alternative to DE analysis to build a reliable set of determinant features (*i.e.*, genes) characterising each group, preserving also non-linear relationships.

The aim of this short paper is to provide a minimal proof-of-concept example of the above-described limitation, which has never been reported so far, suggesting and highlighting the strength of adopting XAI-based alternatives. This is done by considering three well-motivated synthetic datasets (see Discussion), each consisting of two groups, where features mimic the values of a gene-expression matrix, and some of them result in linear or non-linear relationships with groups. Firstly, we analysed the groups with DE analysis, showing that only those features corresponding to a linear separation between groups emerge as significative; secondly, we used simple models distinguishing between the two groups to apply XAI-based explanations and proving that, in this case, also features having non-linear relations with groups are detected as relevant.

Methods

Datasets

We built three synthetic datasets, named (A) clouds, (B) circles and (C) circles (big), respectively. Datasets A and B (see Figure 1) are made of 20 variables (x1, …, x20 – also referred to as features), mimicking the values of a gene expression matrix where each feature represents a gene. Out of the 20 variables, 18 are built as pure noise, sampling the values from uniform distributions. The remaining two variables for each dataset are instead "significative", allowing to distinguish well-separated groups. "Significative" variables have been set randomly by drawing two numbers between 1 and 20 with uniform probability for each dataset. C is similar to the others in that it is characterised by two "significative" variables, but it is meant to prove that the number of variables involved and correlations between them are actually irrelevant to the issue considered. As such, it is made of additional 800 noisy variables, plus 198 other variables with linear correlations with the others (correlation coefficients have been computed and found to be variable up to 0.99). Overall, the third dataset is made of 1000 variables with a signal-to-noise ratio of 0.002.

Each of the three datasets consists of 2000 points, equally divided into two groups of 1000 that are meant

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to represent two subtypes or classes of interest within the dataset, in the case of clustering and classification models, respectively. Considering the two significant variables, the groups are generated as follows:

(A) Clouds. Bivariate Normal distributions with different centres and same standard deviation in both directions. This dataset is exemplary of a linear relation between significant features and groups. From a biological perspective, it represents, for example, a couple of genes that are over- and under-expressed in the two groups, respectively.

(B) Circles. The inner cloud is sampled from a bivariate Normal distribution, whereas the outer cloud is sampled from a Gamma distribution with an offset on the radial coordinate. This dataset is exemplary of a purely non-linear relation between significant features and groups. From a biological perspective, it represents, for example, a couple of genes whose expression has to be kept in homeostasis for health conditions, and a disbalance of expression levels causes the behaviour of interest.

(C) Circles (big). The two significant variables are sampled in the same way described for dataset B, thus defining a similar circular pattern that has not been shown in the figure to avoid redundancy. The difference with respect to dataset B lies in the number of noisy variables and the presence of correlations between variables, as previously described.

Moreover, we built a supplementary dataset D (see Supplementary Materials[1]), similar to dataset C but with an increased number of significant variables and synthetic expression values sampled from negative binomial distributions mimicking those of a real RNA-seq dataset (see Supplementary Figure 3[1]). This supplementary dataset is meant to show that the number of significant variables and the underlying distributions are not affecting the results hereafter presented.

Differential expression analysis

For each of the three datasets, we carried out a differential expression analysis between groups. Computations have been performed using two different algorithms, namely DESeq (Love *et al.*, 2014), implemented in the R package DESeq2, which uses shrinkage estimation for dispersions and fold change estimates, and GLM (McCarthy *et al.*, 2012), implemented in the R package edgeR, which applies a kernel transformation to the feature space before regression. Genes are considered significantly differentially expressed in the case of an adjusted p-value below 0.05.

Machine Learning models

For each of the three datasets, we built a simple model distinguishing between the two groups and providing the labels reported in Figure 1 (*i.e.*, tagging "red" and "blue" samples). In particular, for dataset A (clouds) we used a linear model implementing a decision boundary lying on the significant variable plane and perpendicular to a line passing through the centres of mass of the two clouds. For dataset B (circles) and C (circles – big), we used a non-linear model implementing a circular decision boundary lying on the significant variable planes, respectively, centred on the centre of mass of the inner cloud. Implementation is public and available on GitLab (see Data & code availability section).

XAI-based explanation analysis

We applied an XAI-based approach, training two different explainers for each dataset to interpret the model's output. To this end, we used both LIME (Ribeiro *et al.*, 2016) and kernel-SHAP (Lundberg and Lee, 2017), which are the two most popular explainers for models built on tabular data, and they are both local explainers, meaning that they provide local explanations for each point of the dataset. In particular, the explainers result in a value associated with the importance of each feature in explaining the output of each point. Intuitively, this is done by: **(i)** creating a neighbourhood of the data point to be explained. Such process is different between the two explainers since LIME applies a Gaussian perturbation to the point, whereas kernel-SHAP substitutes some of the values of the features with those sampled from a background set provided as input (we used 20 random points of the dataset). **(ii)** Assigning labels to the neighbours applying the model and; **(iii)** fitting a linear model on the whole neighbourhood that represents a local linear approximation of the global decision boundary between groups in the neighbourhood of the data point to be explained. The weights of the linear approximation are used to assign a local importance score to each feature. The overall importance of the features can be obtained either by averaging the contributions over all the points of the datasets or by considering the median value of the distribution. It should be noted that absolute importance values provided by LIME and kernel-SHAP are not directly comparable.

Data and code availability

The data generated to support the presented findings, as well as the code used for data generation, data analysis and plots, are publicly available on GitLab at: https://gitlab.com/deflect-public/differential-expression/.

Results

The variables randomly selected as significant were x9 and x20 for dataset A, x4 and x17 for dataset B and x23 and x83 for dataset C. Results of the DE analysis for all three datasets are shown in Figure 2. From the adjusted p-value, it results that DE analysis (both DESeq and GLM) is effective in identifying important features in the case of dataset A (clouds), in which x9 and x20 are characterised by $p<0.001$ and noisy features are rejected. Instead, it fails when non-linearity is introduced, *i.e.*, with datasets B (circles) and C (circles – big) in which no significative features are detected.

[1]http://journal.embnet.org/index.php/embnetjournal/article/downloadSuppFile/1035/1035_supp_1

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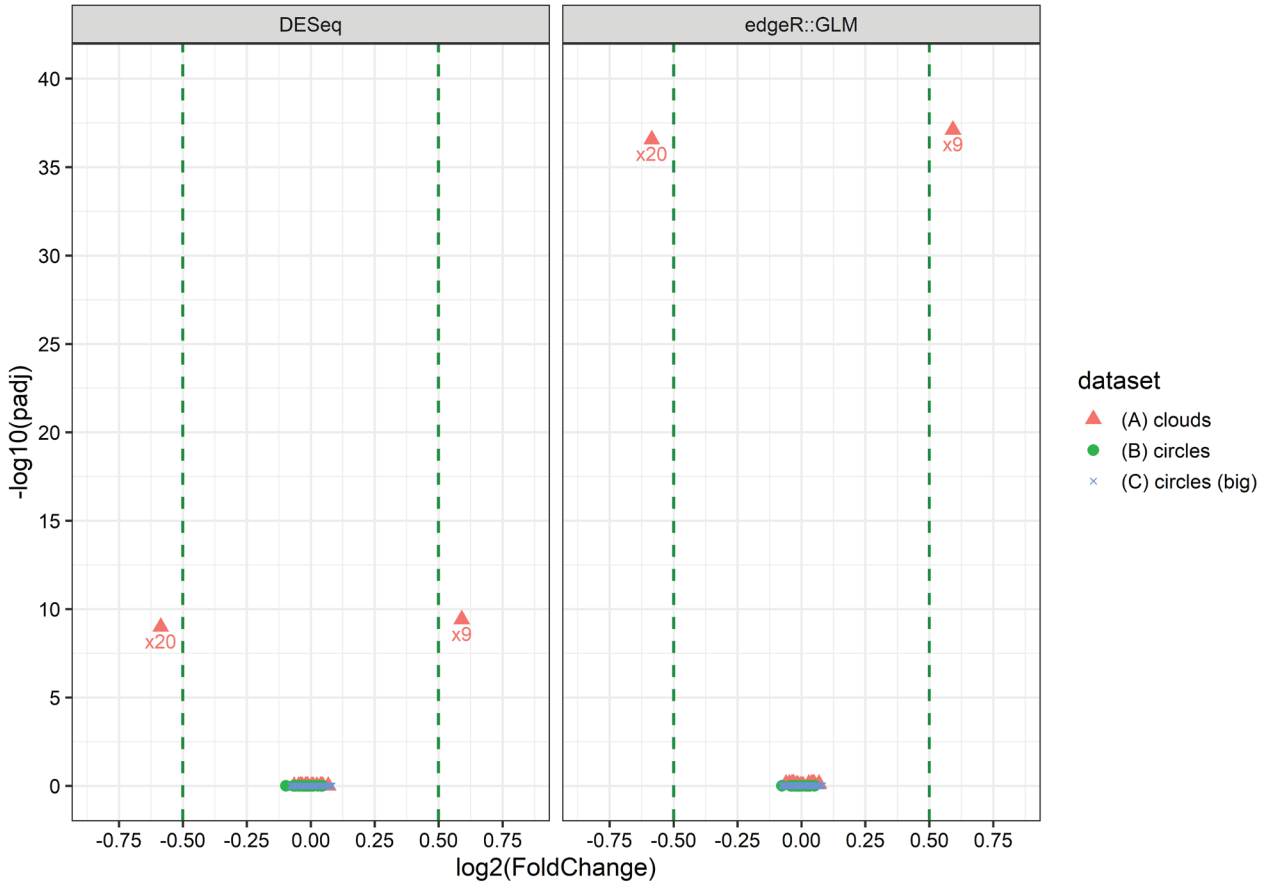

Figure 2. Volcano plot representing the results of differential expression analysis for the three datasets (different colours and shapes) obtained with DESeq (left) and edgeR-GLM (right), respectively. Vertical dashed lines represent significance thresholds. Adjusted p-values have been computed using the Benjamini-Hochberg correction.

Results of XAI-based feature importance analysis are summarised in Figure 3 for both tested explainers, namely LIME and kernel-SHAP. As expected, both the explainers proved to be effective in highlighting the relevant features for all the datasets considered by assigning importance scores way above those of noisy variables.

In the chosen examples, LIME seems to result in distributions that are a bit better separated from noisy variables with respect to kernel-SHAP. On the other hand, kernel-SHAP seems to be more efficient in recognising noisy variables whose contribution is set to zero, whereas LIME typically assigns negligible but non-zero contributions to those variables.

Discussion

The present study focuses on a general issue arising any time ML models are applied to gene expression data to stratify patients based on their molecular profile. In particular, the clustering model or classifier assigns labels to the samples, and researchers have to understand whether the resulting grouping is biologically meaningful or not. This latter process, often referred to as "downstream analysis", involves many further analyses such as GSEA (or pathway analysis) and wet lab validation, and it is possibly followed by clinical validation if the results are considered significant and robust enough. All these analyses, however, rely on the common issue of identifying the subset of genes that have been determinant for the model in assigning the labels or, in other words, the set of genes characterising the groups to be used for subsequent pathway analysis and validation. Such characterisation is very often performed with DE analysis between groups; however, notably, the above-presented findings highlight an intrinsic limitation of DE analysis. In particular, it is very effective if the groups under consideration are (nearly) linearly separable, whereas it fails in identifying relevant features when non-linearity is introduced. This does not mean that DE analysis is wrong, nor it is the intention of the authors to make criticisms of specific previous literature, but that its use as a group characterisation and gene selection method for downstream analysis should be considered with extreme care if coupled with non-linear ML models. Such an aspect, to the best of our knowledge, has not been addressed so far in the literature.

The major implication of this DE limitation is that features that are determinant for the model to distinguish

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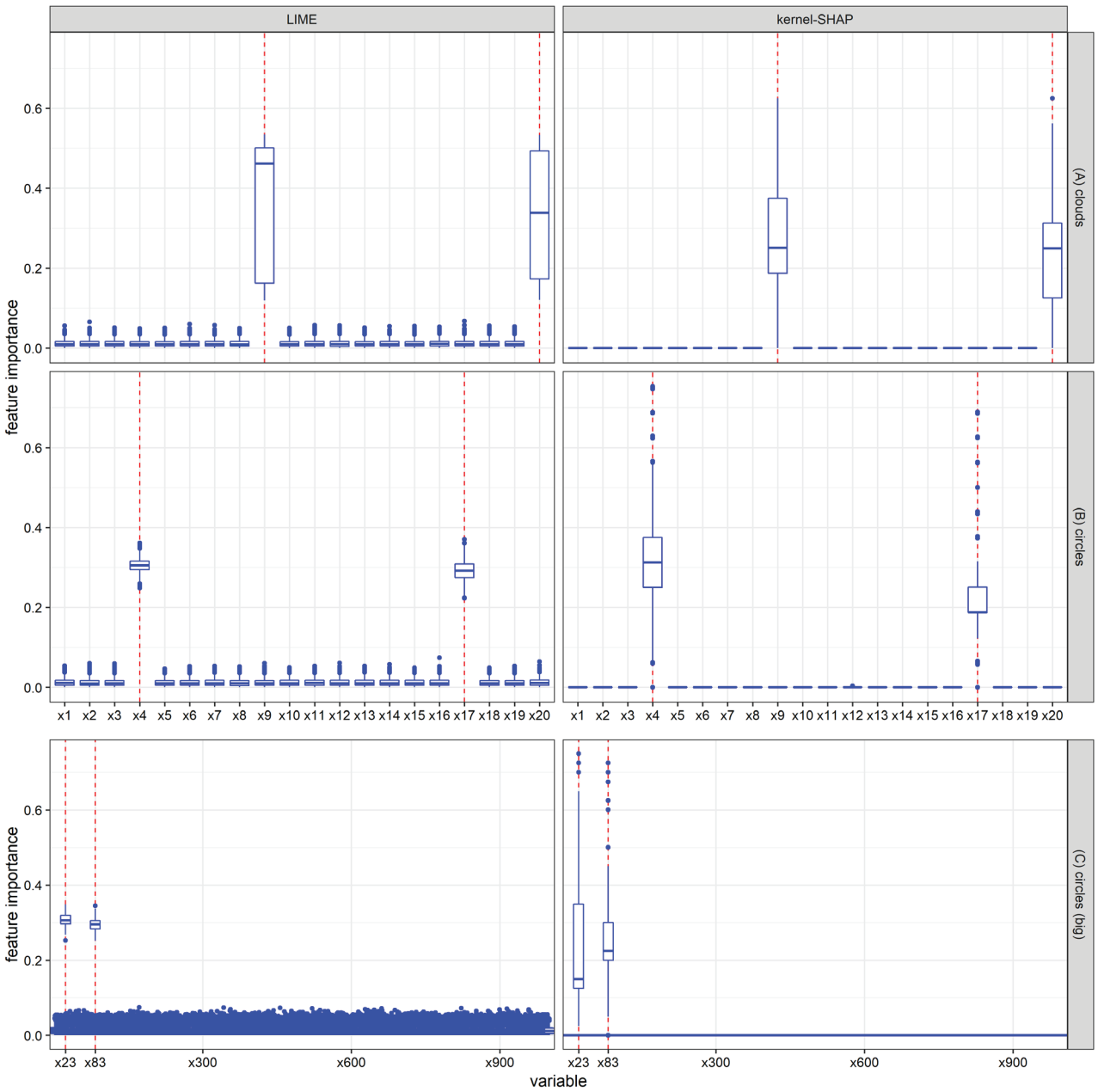

Figure 3. Feature importance of the three datasets (rows) computed using two different explainers, namely LIME (left column) and kernel-SHAP (right column). Boxplots report feature importance based on local explanations of each sample. Vertical dashed lines indicate the relevant features of each dataset.

between the two groups could potentially pass undetected. This has the consequence of affecting the list of genes used for lab validation or pathway analysis, thus potentially compromising the significance of such validation techniques. In other words, if the hypothesis developed is good, it may be that relevant biological characteristics of the groups under consideration evade the attention of researchers and limit the interpretability of results or, worse, that the hypothesis is rejected because results are erroneously considered not biologically meaningful.

Concerning the model, we would like to insist that the considerations made throughout the paper are valid both for classifiers and clustering models. In fact, for our purpose, the model might be considered as a black-box tool that, given the input data, provides labels as output, establishing a linear or non-linear relationship between inputs and output. Instead, we propose that the focus should lie on methods that allow to understand which features were actually relevant in determining such labels. As a consequence, details on classification accuracy or model training are not provided since they are not pertinent for clustering models and, overall, irrelevant to the results and conclusion presented.

As a complement to DE analysis, we propose to adopt (whenever possible) an XAI-based approach, which we

demonstrated to be able to overcome limitations related to non-linearity. On the other hand, such an approach has some drawbacks that should be considered. First, it is not always applicable, especially because it requires that the AI model selected can be used to predict labels of new data points. But, this is only the case of supervised models and the subset of unsupervised models that create a partition of the feature space, such as kMeans-based models. For the other cases, DE analysis still remains the only option. Second, research on XAI models is a cutting-edge topic in the field of ML applications, and it is still in its early years. Thus, little guidance exists in order to help researchers in choosing the best configuration of parameters to get reliable estimations of feature importance from the explainers, which at this point requires tuning of hyperparameters and their combinations. In this direction, it is worth mentioning the work of (Amparore *et al.*, 2021) in providing reliable metrics to quantify the quality of XAI explanations, which may be helpful in guiding hyperparameter tuning. Finally, while DE analysis provides p-values associated with fold change estimates, a major limitation of the XAI-based approach is that it only provides a number whose absolute value is associated with feature importance. As a result, relevant and not relevant features have to be defined by means of a threshold, typically applied to the average importance value, which may not be straightforward to set either since there is not a general analytical rule. A possible method would be to look for gaps or "knees" in the ordered feature importance plot.

A further element that is worth discussing is our choice of simulated data instead of real data, which is generally preferred in methodological studies related to gene expression. Indeed, in this case, the choice is well motivated by several considerations. First, simulated data allow us to control which genes are relevant for classification and to verify if they are actually detected by the approaches considered without any dependencies on the biological interpretation of results, which instead would not have been possible with a real dataset. Plus, many biological interpretations are based on existing methodological results and thus subject to their shortcomings: using them would have resulted in a self-feeding vicious circle. Second, it is true that real datasets are characterised by many genes interacting with each other, but we show with dataset C that the number of variables and correlations between features does not affect the methods considered, apart from increasing the computing time, and that the conclusions derived from dataset A and B, still hold for dataset C. In fact, results for dataset C, characterised by numerous and correlated features, are equivalent to those obtained with dataset B (see Figures 2 and 3), characterised by few features without correlations. Moreover, with supplementary dataset D[1], we also show that the number of significant variables or the underlying distribution of the synthetic expression values do not affect the results. Third, and probably most importantly, we highlighted how DE limitations arise in the case of non-linear relations between gene expression data and the resulting groups. It is important to note that such grouping is the output of the model (*i.e.*, labels), so that non-linearities are introduced by the model itself and do not necessarily coincide with the underlying "ground truth". In other words, what is actually relevant is not the structure of the dataset, but rather the shape of the decision boundary defined by the model. If the model results in a non-linear decision boundary, which is likely to happen when complex ML models are used, DE analysis may not be effective in identifying the relevant variables for group characterisation. Conversely, as we have shown, the XAI-based approach is better suited, independently of the dataset. Finally, we stress that the focus of this study is purely methodological; thus, although a real dataset would have been illustrative of a full downstream analysis leading to a biological interpretation based on the relevant features (genes) detected, in this case, we are just assessing the capability of each method to detect those relevant features under different conditions.

It should be noted that while the present study focuses on differential expression, the underlying idea has a broader application, and there are no conceptual limitations in extending it to similar analyses under the same assumptions, such as the case of group characterisation based on differentially methylated genes (see, *e.g.*, Kolbe *et al.*, 2014).

As a final remark, we would like to point out that, with this study, we are not presenting an original XAI model and that there may be better XAI-based approaches to use as a complement to DE analysis, depending on the specific application. Nonetheless, the limitation of DE analysis that we are highlighting still holds and should be considered when characterising the biology of groups identified with non-linear ML models.

Conclusions

In conclusion, in the present study we identified a potential limitation of DE analysis used as a gene selection method for subsequent enrichment analysis and lab validation when patient grouping is obtained with the application of complex non-linear ML models. We provided a proof-of-concept example of such limitation by exploiting three synthetic datasets. To overcome the issue, we suggest using XAI-based alternatives that can be effective on the cases considered.

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Key Points

- DE has limited capability to detect non-linear relationships between features and target.
- DE limitation becomes relevant when coupled with complex (non-linear) ML clustering or classification models.
- For the subset of ML models that can predict labels for new data points, DE limitation can be overcome by applying XAI to interpret ML output and detect the relevant features.

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Research Papers

Validation of the Greek Version of Social Appearance Anxiety Scale in Adolescents and Young Adults

Triada Konstantina Papapanou[1], Flora Bacopoulou[2]✉, Maria Michou[3], Christina Kanaka-Gantenbein[1], Dimitrios Vlachakis[4], George P. Chrousos[5], Christina Darviri[1]

[1]Postgraduate Course of Stress Science and Health Promotion, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[2]Center for Adolescent Medicine and UNESCO Chair on Adolescent Health Care, First Department of Pediatrics, School of Medicine, National and Kapodistrian University of Athens, Aghia Sophia Children's Hospital, Athens, Greece

[3]Human Ecology Laboratory, Department of Home Economics and Ecology, Harokopio University, Kallithea, Athens, Greece

[4]Genetics Laboratory, Biotechnology Department, School of Applied Biology and Biotechnology, Agricultural University of Athens, Greece

[5]University Research Institute of Maternal and Child Health & Precision Medicine and UNESCO Chair on Adolescent Health Care, Medical School, National and Kapodistrian University of Athens, Aghia Sophia Children's Hospital, Athens, Greece

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Abstract

Many people are worried about their social appearance. The fear of negative evaluation and judgment regarding one's look in social circumstances is referred to as social appearance anxiety. Social appearance anxiety belongs to social anxiety. The aim of the present study was to validate the Social Appearance Anxiety Scale (SAAS) in the Greek language and to examine its psychometric properties. An online survey was conducted in a Greek population sample of adolescents and young adults aged 18 to 35 years. The survey instruments included the Social Appearance Anxiety Scale, the Social Physique Anxiety Scale (SPAS), 2 subscales of Multidimensional Body-Self Relations Questionnaire Appearance Scale (MBSRQ), the Appearance Schemas Inventory-Revised Scale (ASI-R) and the Depression Anxiety Stress Scale (DASS). A total of 429 respondents participated in this research. The statistical analysis showed that the Greek version of the SAAS has good psychometric properties. The internal consistency of questions within the SAAS was 0.942. Positive correlations were found between SAAS and SPAS, the overweight preoccupation subscale of MBSRQ, the ASI-R and the DASS, while negative correlations were observed between SAAS and the appearance evaluation subscale of MBSRQ and age. The results of this study suggest that the Greek version of SAAS can be used as a reliable and valid instrument in the Greek population

Introduction

Social anxiety disorder consists of/includes several types of social fear, such as social interaction anxiety and fear of negative evaluation of the appearance. Most studies focus on social physique anxiety (Levinson and Rodebaugh, 2011). The meaning of Social Appearance Anxiety is a subtype of social anxiety and refers to the concern for the negative evaluation of appearance and the fear of rejection by others due to appearance (Hart *et al.*, 2008; Claes *et al.*, 2011). The anxiety about social appearance is caused by the idea that people cannot make a positive impression on other people (Leary and Kowalski, 1995) and is a result of their negative perception of the body and appearance. Social appearance anxiety is a kind of social anxiety and is defined as the anxiety and intensity that people experience when their external appearance is evaluated by other people (Haydar Şar, 2012). These people are therefore characterised by introversion, pessimism, insecurity, inadequacy in social relationships, keeping their distance and avoiding others and constantly wait for approval and acceptance from their environment. They also try to hide their body or parts of their body that they do not like. The social, academic and professional sectors can be negatively affected by social appearance anxiety (Baltaci *et al.*, 2021). Social appearance anxiety includes a more complete concept of physical appearance, extending from the general physical characteristics associated with physiques such as height, weight and muscle structure to more personal characteristics such as skin, hair, face shape and size of features (Argon, 2014), therefore, the term broader as it

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includes both general appearance problems and physique concerns. (Hart *et al.*, 2008; Levinson and Rodebaugh, 2011). Studies have found correlations between the social appearance anxiety and eating disorders, the fear of negative evaluation (Levinson and Rodebaugh, 2011; Brosof and Levinson, 2017; Hart *et al.*, 2015; Levinson *et al.*, 2013), the avoiding of social relationships, the feelings of loneliness and dependence on social media and the internet (Ayar *et al.*, 2018; , Dogan and Çolak ,2016), the self-esteem, the body image (Claes *et al.*, 2011; Demirel, 2019), the negative perception of body image (Hart et. al., 2008), dissatisfaction with body image (Baratelli, 2009; Boersma and Jarry, 2013; Dakanalis *et al.*, 2016), body mass index and perfectionism (Levinson *et al.*, 2013). Body image is a subjective experience and depends on how the person interprets oneself. How a person perceives his body shows how he perceives himself (Dixit and Luqman, 2018). Many are worried about some part of their body. Concerns about body image show up in childhood and are especially strong in adulthood (Quittkat *et al.*, 2019). According to Sabiston *et al.*, negative body image and physical dissatisfaction can lead to the presence of social appearance anxiety (Sabiston *et al.*, 2007). Furthermore, body checking behaviors occur when someone control or monitor possible changes in weight or body shape (Shafran *et al.*, 2004). According to Claes (2011), social appearance anxiety showed positive correlations with the negative body image and social anxiety. A study in German population found a positive correlation of the SAAS scale with measures of social anxiety and disorders related to body image, such as greater concern for diet, weight and body shape, control of the body (body checking) and avoiding focus on the body (body avoidance). A weak positive correlation with BMI was also found (Reichenberger *et al.*, 2021).

There are some measures that are used for social anxiety such as the Social Physique Anxiety Scale (Hart *et al.*, 1989), the Liebowitz Social Anxiety Scale (Liebowitz, 1987), and the Social Phobia Inventory (Argyrides *et al.*, 2014). These scales are focused on more particular characteristics of appearance, hence questionnaires that assess more general characteristics of appearance are needed. The social appearance anxiety scale (SAAS) is a self -reported measure with 16 items designed by Hart *et al.* (Hart *et al.*, 2008). This scale has been translated and validated in various languages such as Turkish (Doğan, 2010), Persian (Iranian) (Goodarzi *et al.*, 2021), Italian (Dakanalis *et al.*, 2016), German (Reichenberger *et al.*, 2021), Portuguese (Donofre *et al.*, 2021), with good psychometric properties. The purpose of this research was to validate the SAAS in the Greek language and to assess its psychometric properties in a sample of adolescents and young adults aged 18-35 years, in Greece.

Materials, Methodologies and Techniques

Translation procedure

In the present study and validation of scale, the methodology of translation and intercultural adaptation was followed. The instrument was freely available, and no special permission for its use and validation was needed. Two independent individuals, who were Greek native speakers with an advanced level of English/very good knowledge of English, translated the scale from English to Greek (forward translation). Comparison of the two translations resulted in the first version of the scale in Greek and then one bilingual individual translated it from Greek into English (backward translation). The back-translation was compared with the original scale to record any disagreements. Subsequently, the Greek version was administered to 16 people for a test-retest to identify unclear points, to make the necessary corrections, and to determine its final version.

Participants and procedures

The questionnaires were distributed online using Google Forms through various social media platforms such as Facebook, Instagram, Viber, and e-mails. Study participants were undergraduate or postgraduate students aged 18-35 years, employees or unemployed who were able to read and write in the Greek language. People with a diagnosis of severe mental disorder under medication were excluded. Data were collected online from the 2nd of March until the 30th of June 2022.

Ethical considerations

The study's protocol was approved by the scientific committee of the "Science of Stress and Health Promotion" Master's Program and the Bioethics Committee of the Medical School of the National and Kapodistrian University of Athens (protocol number 611/25.02.2022). Participants were fully informed about the aims of the study through a brief text of the study's protocol and were asked to tick a square to indicate their consent to participate in the research. The submission of their response was considered as online consent. Participants were also informed that they could leave the research whenever they wanted. The completion of the questionnaires was anonymous and voluntary, with the ability of withdrawal and termination at any time, without any consequences. The completion time was approximately 15 minutes.

Measures

Sociodemographic Questionnaire

Participants completed information such as sex, age, height, weight, marital status, educational level, work status, social media use and time of use (hours per day).

Social Appearance Anxiety Scale (SAAS)

It is a self-report scale designed by Hart *et al.*, in 2008, which consists of 16 items. It measures the anxiety that is created when someone is negatively evaluated and judged by others because of one's overall appearance, including body shape. It is used a 5-point Likert scale with a range from 1 (not at all) to 5 (extremely). The first item is reverse-coded. The range score is between 16 to 80. Higher scores in this scale mean high level of social appearance anxiety. It consists of a single factor and has no subscales. Cronbach's coefficient was reported as 0.94, 0.95, 0.94 for the three samples in the original study. An example of the items includes "I feel comfortable with the way I appear to others" (Hart *et al.*, 2008).

Social Physique Anxiety Scale (SPAS)

This is a 12-item scale, without subscales, that measures the level of anxiety that a person experiences when realises that others evaluate or may negatively evaluate the physique, the anxiety about physical appearance, when the person is in an environment where he considers that his/her body is subject to evaluation by others. The scale is focused on physique-related issues such as body fat, muscle tone, body proportions and does not include other related areas of appearance anxiety. Each item is answered on a 5-point Likert scale ranging from 1 (not at all) to 5 (too much) (Hart *et al.*, 1989). The reliability index of the Cronbach a was 0.85 and the reliability coefficient of examination-re-examination was r=0.84. The questionnaire has been translated and validated in the Greek language (Psychountaki *et al.*, 2004).

Multidimensional Body-Self Relations Questionnaire Appearance Scale (MBRSQ-AS)

This is a 34-item questionnaire that evaluate attitudes / beliefs for body image. Every item is rated on a 5-point Likert scale ranging from 1 (definitely disagree) to 5 (definitely agree). This edition is a short version of a widely used 69-item questionnaire. It consists of 5 subscales but in our research will be used 2 of the 5 subscales.

Appearance evaluation subscale (7 items)

This subscale evaluates feelings of physical attractiveness or unattractiveness. High scores indicate greater positive feelings and satisfaction with the appearance of the body (Cash, 2000b).

Overweight Preoccupation subscale (4 items)

This subscale assesses one's anxiety about weight, weight control or vigilance, dieting and nutritional restraint (Cash, 2000b). It has been validated in the Greek language with good psychometric properties (Argyrides *et al.*, 2013).

Appearance Schemas Inventory-Revised (ASI-R)

The first version consists of 14 items, but after some changes the final version consists of 20 items (revised ASI-R), which include 2 subscales. Items are answered on a five-point scale ranging from 1 (strongly disagree) to 5 (strongly agree). This scale evaluates the beliefs about the importance meaning and effects of appearance on one's life. Also, it evaluates the malfunctioning shape of the body image (Cash and Labarge, 1996).

Self-Evaluative Salience (12 items)

This subscale refers to the way people believe that their own self-worth could be determined by their physical appearance.

Motivational Salience (8 items)

This subscale concerns an individual's commitment with their appearance, such as grooming behaviors (Cash, 2003).

The ASI-R has been found to have good psychometric properties and it has been validated in the Greek Language (Kkeli and Argyrides, 2013).

Depression Anxiety Stress Scale – 21 items (DASS-21)

It is a self-report questionnaire and consists of three self-administered scales, which designed to measure depression, anxiety and stress. Each of the subscales consists of 7 questions, 21 total questions. A 4-point Likert scale is used from 0 (did not apply to me at all) to 3 (applied to me too much or most of the time). It has been validated in the Greek language and is considered a reliable and valid tool for measuring depression, anxiety and stress in non-clinical population (Pezirkianidis, 2018)

Statistical analysis

Data are presented as frequencies (%) for the categorical variables and means, standard deviations (SD), median and interquartile range (IQR) for continuous variables. The sample adequacy and correlation between items were assessed with the Kaiser-Meyer-Olkin (KMO) statistic and Barlett's Test of Sphericity. The factors identification of the SAAS was done by the Principal component analysis (PCA). Cronbach's alpha values were calculated to examine the internal consistency of the questionnaire. Correlations between the SAAS and other measurements of the study were calculated. Due to the fact that the normality of data distribution was violated, non-parametric Spearman's rho coefficient was conducted to examine correlations between quantitative variables and the SAAS. Non-parametric Mann-Whitney U and Kruskal-Wallis tests were used to evaluate between-group differences. Statistical analyses were performed using IBM SPSS version 24.0 for Windows.

Results

The sample consisted of 429 participants from the general adolescent and young adult population in Greece. Participants' sociodemographic characteristics and measurements are presented in Table 1. The median age was 25 years (IQR=6). Most participants were females (69.2%), unmarried (91.6%), with a Bachelor's Degree (41.7%) or high school education (34.5%) and most of

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Table 1. Participants' sociodemographic characteristics and measurements.

Sociodemographic characteristics N=429		Scales and subscales scores		
Sex N (%)		SPAS score	Median (IQR)	31.00 (13.00)
- Women	297 (69.2%)		Mean (SD)	31.87 (9.10)
- Men	132 (30.8%)			
Age Median (IQR)	25.00 (6.00)	MBSRQ score		
Mean (SD)	25.30 (4.62)	- Appearance evaluation	Median (IQR)	26.00 (7.00)
			Mean (SD)	25.18 (5.33)
Marital status N (%)				
- Married	36 (8.4%)	- Overweight preoccupational	Median (IQR)	9.00 (5.00)
- Unmarried	393 (91.6%)		Mean (SD)	9.54 (3.69)
Education level N (%)		ASI-R Score		
- Until High School	148 (34.5%)	-Self evaluative salience	Median (IQR)	35.00 (12.5)
- Higher Education Institution/	179 (41.7%)		Mean (SD)	34.80 (9.13)
Technological Educational Institute		- Motivational Salience	Median (IQR)	27.00 (8.00)
- College	17 (4%)		Mean (SD)	26.73 (5.60)
- MSc-PhD	85 (19.8%)			
Job Status N (%)		DASS Score		
- Public Employee	48 (11.2%)	-stress	Median (IQR)	8.00 (8.00)
- Private Employee	128 (29.8%)		Mean (SD)	8.40 (5.76)
- Freelance	49 (11.4%)			
- University Student	151 (35.2%)	-anxiety	Median (IQR)	4.00 (9.00)
- Unemployed	41 (9.6%)		Mean (SD)	5.83 (5.60)
- Other	12 (2.8%)			
		-depression	Median (IQR)	5.00 (9.00)
			Mean (SD)	6.79 (6.12)

them were either university students (35.2%) or private employees (29.8%).

Table 2 shows the results of the Principal Component Analysis (PCA) of the 16 items with orthogonal rotation (varimax). The adequacy of the sample was examined using the Kaiser-Meyer-Olkin test and the KMO coefficient was 0.948. Barlett's test of sphericity $\chi2(120) = 4795.374$, $p<0.0001$, showed that correlations between items were sufficiently large to perform PCA. Only one component had eigenvalue >1 which explained

Table 2. Rotated factor loadings of the principal components analysis (PCA) for 16 items of SAAS (N= 429).

Item	SAAS
1. I feel comfortable with the way I appear to others.	0.551
2. I feel nervous when having my picture taken.	0.456
3. I get tense when it is obvious people are looking at me.	0.502
4. I am concerned people won't like me because of the way I look.	0.767
5. I worry that others talk about flaws in my appearance when I'm not around.	0.729
6. I am concerned people will find me unappealing because of my appearance.	0.830
7. I am afraid people find me unattractive.	0.818
8. I worry that my appearance will make life more difficult for me.	0.770
9. I am concerned that I have missed out on opportunities because of my appearance	0.727
10. I get nervous when talking to people because of the way I look.	0.743
11. I feel anxious when other people say something about my appearance.	0.773
12. I am frequently afraid that I won't meet others' standards of how I should look.	0.794
13. I worry people will judge the way I look negatively.	0.874
14. I am uncomfortable when I think others are noticing flaws in my appearance.	0.786
15. I worry that a romantic partner will/would leave me because of my appearance	0.774
16. I am concerned that people think I am not good looking.	0.850
Eigenvalues	8.842
% of Variance	55.26

Table 3. Range, mean, standard deviation (SD), item-total correlation and Cronbach's alpha coefficient of the SAAS scale.

Scale	Range	Mean (SD)	Min-Max	Item-total correlations	Alpha of scale
SAAS	16-80	33.92 (13.76)	16-79		0.942
1. I feel comfortable with the way I appear to others.				0.502	
2. I feel nervous when having my picture taken.				0.424	
3. I get tense when it is obvious people are looking at me.				0.469	
4. I am concerned people won't like me because of the way I look.				0.725	
5. I worry that others talk about flaws in my appearance when I'm not around.				0.681	
6. I am concerned people will find me unappealing because of my appearance.				0.791	
7. I am afraid people find me unattractive.				0.778	
8. I worry that my appearance will make life more difficult for me.				0.720	
9. I am concerned that I have missed out on opportunities because of my appearance				0.668	
10. I get nervous when talking to people because of the way I look.				0.697	
11. I feel anxious when other people say something about my appearance.				0.736	
12. I am frequently afraid that I won't meet others' standards of how I should look.				0.750	
13. I worry people will judge the way I look negatively.				0.846	
14. I am uncomfortable when I think others are noticing flaws in my appearance.				0.753	
15. I worry that a romantic partner will/would leave me because of my appearance				0.728	
16. I am concerned that people think I am not good looking.				0.814	

55.26% of the total variance and all factor loadings were >0.45. The factor load of the items was within the range of 0.456-0.874.

Table 3 presents descriptive statistics for the SAAS, such as range, mean, standard deviation, item - total correlation and Cronbach's alpha coefficient. The score range of the scale was 16-80, the mean (SD) was 33.92 (13.76). The reliability of the SAAS scale was investigated by internal consistency according to the Cronbach's a. The value of the coefficient was 0.942.

The score of participants in the SAAS scale was correlated with other scales. As shown in Table 4, there were statistically significant correlations with all scales with $p<0.0001$. SAAS showed weak positive correlations with the subscale Motivational Salience of the ASI-R (r=0.208) and with the subscale Overweight Preoccupation (r=0.291) of the MBSRQ, moderate positive correlations with the subscale for the anxiety (r=0.404), the stress (r=0.442) and the depression (r=0.451) of the DASS, moderate to strong positive correlations with the SPAS (r=0.791) and the subscale of Self-Evaluative Salience of ASI-R (r=0.659). Furthermore, a negative correlation was found with the subscale Appearance Evaluation of the MBSRQ (r=-0.600).

Table 4. Correlations (Spearman's rho) between SASS and other study measurements.

	Age	SPAS	MBSRQ Appearance Evaluation	MBSRQ Overweight Preoccupation	ASIR Self Evaluative salience	ASIR Motivational Salience	DASS Stress	DASS Anxiety	DASS Depression
Spearman rho	-0.123	0.791	-0.600	0.291	0.659	0.208	0.442	0.404	0.451
p-value	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

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Table 5. Associations between the SAAS scale and other study measurements.

Study measurements	Categories	SAAS Median (IQR) Mean (SD)
Sex Median (IQR) Mean (SD)	Males	25.00 (17.00)
		30.06 (13.16)
	Females	33.00 (18.00)
		35.63 (13.69)
	p-value	<0.0001
Education level Median (IQR) Mean (SD)	Until High School	31.00 (22.50)
		35.14 (15.76)
	Higher Education Institution/Technological Educational Institute	30.00 (20.00)
		33.22 (13.25)
	College	35.00 (16.50)
		35.53 (11.95)
	MSc-PhD	30.00 (16.00)
		32.93 (11.19)
	p-value	0.742
Marital status Median (IQR) Mean (SD)	Married	27.50 (16.25)
		29.83 (13.01)
	Unmarried	31.00 (19.00)
		34.29 (13.78)
	p-value	0.028
Job status Median (IQR) Mean (SD)	Public Employee*	28.50 (17.50)
		30.13 (10.05)
	Private Employee^	28.50 (19.00)
		32.50 (13.50)
	Freelance<	27.00 (13.00)
		29.98 (11.85)
	University Student>	34.00 (21.00)
		35.44 (14.54)
	Unemployed * ^ < > -	40.00 (18.50)
		42.54 (14.35)
	Other-	31.00 (18.00)
		31.67 (10.75)
	p-value	<0.0001

*** ^ < > - indicate significant differences between the groups**

Likewise, there was a significant negative correlation between SAAS and the age of participants with r=-0.123 (p=0.001).

Table 5 presents associations between the SAAS and other study variables. There was statistically significant difference in SAAS according to sex, as women scored higher than men (p<0.0001). Also, unmarried participants scored higher than married participants (p=0.028). According to the job status, unemployed individuals scored higher than those who were public employees, private employees, freelancers or university students (p<0.0001).

Discussion

The current study aimed to evaluate the validity and reliability, factor structure and psychometric properties of the Social Appearance Anxiety Scale in the Greek language. The SAAS in our study have demonstrated good internal consistency with Cronbach's alpha= 0,942, which is consistent with the results of the initial research of Hart *et al.* (2008) (a1 = 0.94, a2 = 0.95, a3 = 0.94 for the 3 samples) and with validations in other languages (Reichenberger *et al.*, 2021; Doğan, 2010; Goodarzi *et al.*, 2021; Donofre *et al.*, 2021).

In this study women had higher levels of social appearance anxiety than men. This finding has been

confirmed by other researchers, who found that women had higher score of SAAS than men, and therefore more social appearance anxiety (Reichenberger *et al.*, 2021; Dakanalis *et al.*, 2016; Levinson and Rodebaugh, 2011). In the original research the scores of SAAS were not correlated with female sex (Hart et. al., 2008) and this finding was consistent with other studies in which participants' SAAS scores did not differ considerably according to sex (Amil and Bozgeyikli, 2015; Şahin *et al.*, 2013). In line with other studies, SAAS demonstrated a significant positive correlation with SPAS (Hart *et al.*, 2008; Goodarzi *et al.*, 2021). Additionally, in our study SAAS was positive correlated with measures such as overweight preoccupation, and negative correlated with the appearance evaluation, which is consistent with the results of another validation of the scale (Reichenberg *et al.*, 2021) and the original study (Hart *et al.*, 2008). Furthermore, the results of the validation showed a moderate positive correlation between the SAAS and the DASS. This finding is consistent with the results of previous studies. A study of Malaysian students found that those who had high levels of social appearance anxiety were more likely to have high scores on depressive symptoms (Kadir *et al.*, 2014). In general, positive correlations of social appearance anxiety with depression, anxiety and stress have been found. It is observed that as social appearance anxiety increases, the levels of depression, anxiety and stress also increase (Çelik and Tolan, 2021). Furthermore, people who are worried about their appearance have high scores of depression, anxiety and stress (Öncü *et al.*, 2013). In terms of predictive validity, the SAAS seemed to be the only prognostic factor of depression beyond the indicators of social anxiety and negative body image (Hart *et al.*, 2008).

This study has some limitations. Firstly, it was not possible to perform a test-retest analysis to examine the consistency of the instrument over time due to anonymity. Secondly, the sample mainly consisted of women, limiting the generalisation of the findings. In future studies, it is important to investigate the validity of SAAS factors in different/other samples and to evaluate the convergent and divergent validity with additional measures. Future research could compare the levels of SAAS between psychiatric and non-psychiatric patients and use this tool in clinical populations with social anxiety disorder, eating disorders, physical dysmorphic disorder, orthorexia, other medical conditions that affect appearance such as dermatological diseases or deforming conditions, in younger adolescents and in older adults and the elderly.

In conclusion, the present study indicates that the Greek version of Social Appearance Anxiety Scale has good psychometric properties. The scale refers to the fear of being judged by others based on one's physical appearance, such as body shape. The SAAS can be considered a reliable tool for measuring anxiety of adolescents and young adults in situations where the assessment/judgement of one's appearance is possible.

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Key Points

- Questionnaires for social appearance anxiety in Greece are lacking.
- The Social Appearance Anxiety Scale (SAAS) measures the anxiety created when someone is negatively evaluated and judged by others because of one's overall appearance.
- The Greek version of SAAS was validated in a sample of 429 adolescents and young adults.
- The Greek version of the SAAS demonstrated good psychometric properties and internal consistency.

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Research Papers

Validation of the Eating Habits Questionnaire in Greek adults

Georgia Bali[1], Ioulia Kokka[1], Fragiskos Gonidakis[2], Eleni Papakonstantinou[3], Dimitrios Vlachakis[3], George P. Chrousos[4], Christina Kanaka-Gantenbein[1], Flora Bacopoulou[4]✉

[1]Postgraduate Course of Stress Science and Health Promotion, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[2]Eating Disorders Unit, First Department of Psychiatry, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece

[3]Genetics Laboratory, Biotechnology Department, School of Applied Biology and Biotechnology, Agricultural University of Athens, Greece

[4]Center for Adolescent Medicine and UNESCO Chair on Adolescent Health Care, First Department of Pediatrics, School of Medicine, National and Kapodistrian University of Athens, Aghia Sophia Children's Hospital, Athens, Greece

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Abstract

Healthy eating has gained ground in people's daily lives in modern society. However, an overwhelming preoccupation with healthy eating can lead to a pathological form setting the ground for orthorexia nervosa. This study aimed to validate the Greek version of the Eating Habits Questionnaire (EHQ) in adults 18 to 65 years old. The EHQ evaluates orthorexia nervosa traits. An online survey was conducted among adults of the general Greek population by administrating a battery of self-report instruments. The IPIP Big-Five personality questionnaire, Beck's Depression Inventory, the Obsessive-Compulsive Inventory-Revised, the Bulimic Investigatory Test, the Edinburg BITE, and the Eating Attitudes Test-13 were used. Internal consistency, test-retest reliability, and convergent and criterion validity were examined. A total of 551 adults (92.2% females) voluntarily participated in the study. Results suggest that the Greek version of the instrument has good psychometric properties. Analysis revealed a 3-factor model explaining 48.20% of the total variance. Cronbach's alphas ranged between 0.80 to 0.82, indicating good internal consistency. The test-retest reliability analysis showed no statistically significant difference between the measurements of the first and the post-2 weeks. Correlations with other eating disorder-related constructs were found to be weak to moderate. Body mass index was not significantly correlated with neither of the three EHQ subscales. The Greek version of EHQ is a robust instrument that could be used in clinical practice and research in the field of eating disorders in Greece.

Introduction

The preventive and therapeutic role of healthy eating in numerous diseases of recent times has resulted in an on-growing interest of both scientists and patients (Kiss-Leizer and Rigó, 2019). Despite the positive outcomes of this otherwise beneficial behavior, in certain cases it might be considered as a social trend that leads to extremities (Douma *et al.*, 2021). In other words, when healthy eating becomes obsessive, psychological disturbances may emerge. This overwhelming preoccupation can become the main life-priority, and set the ground for orthorexia nervosa (ON). Borrowed from the Greek language, the prefix -ortho means "correct" (Gleaves *et al.*, 2013). An individual with ON attempts to reach optimum health through strict dietary practices. The central axis is not the quantity of food, but its perceived quality. Among the behavioral patterns exhibited by those with ON, the consumption of solely what is perceived as healthy and "pure" food, the inflexible dietary rules, and specific for each individual rituals during food processing and meal preparation are included. The obsessive nature of ON can lead individuals to pursue even more rigid diets such as veganism, raw foodyism, and fruitarianism (Hayes *et al.*, 2017). Consequently, this fixation on healthy eating results in clinically significant impairments, such as social withdrawal, nutrient deficits, or even severe malnutrition, and subsequently in impaired quality of life. ON has been found to relate with attachment issues (Barnes and Caltabiano, 2017), perfectionism (Brytek-Matera *et al.*, 2020), narcissism (Oberle *et al.*,

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2017), obsessive-compulsive features (McComb and Mills, 2019) and excessive exercise (Rudolph, 2018; Oberle *et al.*, 2018), and less commonly with depressive symptomatology, sex (Strahler, 2019), and body mass index (BMI) (Brytek-Matera *et al.*, 2020).

ON is not included in mental health classification manuals (American Psychiatric Association, 2013). Currently, it falls into the group of Eating Disorders Not Otherwise Specified (EDNOS), as it appears to have similarities with avoidant/restrictive food intake disorder (ARFID), anorexia nervosa (AN) and obsessive-compulsive disorder (OCD) (American Psychiatric Association, 2013). Although standardized clinical diagnostic criteria are not established, researchers of the field identify the pathological fixation with healthy eating, emotional consequences due to self-imposed rules, and functional impairment across various areas of life as the main criteria (Cena *et al.*, 2019). The 'Eating Habits Questionnaire' (EHQ) was developed by David H. Gleaves and colleagues to assess symptoms of orthorexia nervosa. It is a 21-item orthorexia test comprised of three subscales evaluating the individual's knowledge on healthy eating, and detecting emotional attitudes and other problems towards healthy eating, exhibiting good psychometric properties (Gleaves *et al.*, 2013). The present study aimed to translate and validate the Greek version of EHQ in the general population, to obtain a tool for the assessment of adult orthorexia characteristics.

Materials, Methodologies and Techniques

Translation procedure

The translation procedure was initiated after receiving permission by the first author of the EHQ, and followed several steps, as indicated by similar research (Kokka *et al.*, 2021). The questionnaire was translated in Greek by a mental health professional specialized in eating disorders. The translation aimed more at a conceptual rather than a linguistic equivalent. The Greek version of EHQ questionnaire was back-translated by an independent professional translator with no knowledge of the original questionnaire or the purpose of the study. The back-translated version was evaluated by a bilingual speaker to identify any possible inconsistencies and proceed with the final version of the questionnaire. Following, a pre-test it was administrated to a sample of 10 volunteers to examine the clarity of each question. No changes were required and the final Greek version of the EHQ questionnaire was obtained.

Participants and study design

The survey was conducted online via several social media platforms. Researchers aimed to at least 10 responses per item as suggested by previous validation efforts (Costello and Osborne, 2005). A response was considered eligible for analysis if the respondent was 18 to 65 years old and a Greek speaker. Responses with missing values were excluded from analysis. No additional inclusion or exclusion criteria were applied. To examine the test–retest reliability of each scale, 2 weeks later, 31 of the participants completed the 21-item EHQ to assess the repeatability of the scale across time.

Ethical considerations

The study followed the ethical standards of the Declaration of Helsinki. The study protocol was approved by the scientific committee of the "Science of Stress and Health Promotion" Master's Program of the School of Medicine, National and Kapodistrian University of Athens, Greece (45274-31/8/2020). Eligible participants were extensively informed about the study's purpose with a description of the research protocol before completing the questionnaires. Submission of the response was considered an automatic consent. Participation in the study was not compensated. The volunteers participating in the retest reliability procedure were initially informed in detail and agreed to register and provide their emails for further communication.

Measures

Data were collected using a battery of self-report questionnaires and a demographics' form.

Demographics questionnaire: this included sex, age, educational level, marital status, and history of an eating disorder. Data of current weight and height were requested to calculate the body mass index (BMI).

Self-report on weight satisfaction: participants were asked to respond to the degree to which they were satisfied with their current weight on a 3-point scale ranging from 'Not at all' to 'Absolutely'. Weight satisfaction was examined by the question "Are you satisfied with your current body weight?", and responses were given on the same 3-point scale.

Eating Habits Questionnaire (EHQ): EHQ is used for the evaluation of orthorexia nervosa traits. It consists of 21 items answered on a 4-point Likert-type scale (1: False, not at all true, 4: Very true) and examines three different factors; knowledge of healthy eating (5 items), problems associated with healthy eating (12 items), and feeling positively about healthy eating (4 items) (Gleaves *et al.*, 2013).

IPIP Big-Five personality questionnaire: this instrument is used for the identification of personality traits. It consists of 50 items and responses are given on a 5-point Likert-type scale (1=Very Inaccurate to 5=Very Accurate) and examines the five aspects of the personality; extraversion (10 items), agreeableness (10 items), conscientiousness (10 items), emotional stability (10 items), and intellect/ imagination (10 items). The Greek version of IPIP exhibits good construct validity, internal consistency, and concurrent validity (Ypofanti *et al.*, 2015).

Beck's Depression Inventory (BDI): BDI is used for the evaluation of psychological and physical symptoms of depression. It consists of 21 items and total score ranges from 0 to 13 indicating minimal depression, 14 to 19

indicating mild depression, 20 to 28 indicating moderate depression and 29 to 63 indicating severe depression (Beck *et al.*, 1988). The Greek version of BDI exhibits good psychometric properties (Giannakou *et al.*, 2013).

Obsessive-Compulsive Inventory-Revised (OCI-R): OCI-R is used for the evaluation of OCD symptomatology. It consists of 18 items and examines the six subtypes of obsessive spectrum: washing, checking, ordering, hoarding, obsessing and neutralizing. Responses are given on a 5-point Likert-type scale (0=Not at all to 4=Extremely). Scores higher than 30 indicate characteristics of the obsessive-compulsive disorder (Foa *et al.*, 2002). The Greek adaptation exhibits excellent internal consistency and good convergent, and divergent validity (Simos *et al.*, 2019).

Bulimic Investigatory Test, Edinburg (BITE): BITE is applied for the evaluation of bulimic symptomatology severity. It consists of 33 items and two subscales; symptoms, and symptom severity. The cut-off score for the symptom subscale is 15, while for the severity of symptom subscale 5. The instrument has been translated in the Greek language for research purposes (Henderson and Freeman, 1987).

Eating Attitudes Test-13 (EAT-13): EAT-26 is used for the evaluation of maladaptive behaviours and attitudes associated with anorexia nervosa (Garner *et al.*, 1982). The adaptation of the EAT-26 in the Greek population revealed a new 13 item EAT model, in which responses are given on a 6-point Likert-type scale (1=Always to 6=Never). It examines three different factors; dieting (4 items), food preoccupation (6 items), and important others (3 items) (Douka *et al.*, 2009).

Data analyses

Descriptive analyses were used to calculate frequencies (%) for categorical variables and median (IQR) and mean (SD) for continuous variables. Exploratory Factor Analysis (EFA) on item-level was implemented to assess the three-factor structure of EHQ. The Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO) and Bartlett's Test of Sphericity were utilized to examine sample's adequacy and the adequacy of the correlation between items, respectively. Principal component analysis (PCA) with rotated factor loadings was performed to evaluate the internal structure of the measure. Eigenvalues greater than 1 determined the number of subscales. Each factor included only items with loadings greater than 0.3. Cronbach's alpha values were extracted to estimate internal consistency for the three subscales. The Spearman's rho correlation coefficient was used to examine the inter-correlation between the three subscales. Total scores and subscale scores of instruments were calculated and correlated with the EHQ subscales scores. Association between EHQ subscales and other study measurements were performed by Mann-Whitney, Kruskal Wallis and One way ANOVA tests. The Wilcoxon signed-rank test was used to examine the test-retest reliability of the scale across time. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) for Windows (version 25) statistical software (SPSS Inc., Chicago, IL).

Results

The basic demographic characteristics of the participants, as well as the results for all the questionnaires and their subscales are presented in Table 1. A total of 551 adults from the general population participated in the survey, while 508 (92.2%) were females. Mean scores of the questionnaires indicated that the sample demonstrated high levels of OCD symptomatology (>21), tendency to exhibit an eating disorder (>12), possible presence of bulimia, but no disturbing depression levels (<16).

Exploratory factor analysis (EFA)

The rotated factor loadings of the principal components analysis (PCA) with direct oblimin rotation are presented in Table 2. The value of the Kaiser-Meyer-Olkin (KMO) coefficient was 0.880 which verified that the sample was adequate. Bartlett's Sphericity Test ($\chi 2$) was 4056.685, $p<0.0001$, which indicated that correlations between items were sufficiently large to perform PCA. All three subscales had eigenvalues greater than the 1 and in combination explained 48.20% of the variance. All items had factor loadings >0.3.

Cronbach's alpha values were calculated to explore the internal consistency of the EHQ subscales. Results demonstrated good internal consistency with coefficient alpha values ranging from 0.80 to 0.82. Alpha coefficient was 0.80 for 'Knowledge' subscale, 0.82 for 'Problems' subscale, and 0.82 for 'Feelings' subscale. Correlations between the EHQ subscales were moderate and ranged from 0.44 to 0.52 indicating that elements of the subscales were positively correlated to each other, measuring the same construct without excess (Table 3).

Test-Retest Reliability

The descriptive characteristics of the EHQ and the three subscales, from both first and second measurement points are presented in Table 4. Out of 551 respondents who completed the initial test, 21 subjects agreed to a post-2 weeks second testing to examine the test-retest reliability. The Wilcoxon signed-rank test was used to examine the test-retest reliability of the 3 subscales. No statistically significant difference was found between the two measurement points ($p>0.05$ for each subscale).

Convergent and Criterion validity

The associations between EHQ subscales and other study variables are presented in Table 5. Educational level was significantly associated with the EHQ 'Knowledge' subscale, while marital status with the 'Problems' subscale ($p=0.016$ and $p=0.015$ respectively). To evaluate criterion validity, the EHQ was examined in relation to other instruments measuring related constructs; personality traits, depressive symptomatology, OCD symptoms, maladaptive eating behaviours and bulimia traits. From

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Table 1. Participants' sociodemographic characteristics, scales' and subscales' scores.

Sex	**N (%)**	**IPIP score**	**IQR/SD**
- Female	**508 (92.2)**	**- Extraversion**	**33.00 (8.00)**
- Male	**43 (7.8)**		**32.77 (6.15)**
Age Categories	**N (%)**	**- Agreeableness**	**36.00 (4.00)**
- 18-25 years	**73 (13.2)**		**36.19 (3.07)**
- 26-35 years	**234 (42.5)**	**- Conscientiousness**	**35.00 (10.00)**
- 36-45 years	**204 (37.0)**		**34.75 (6.27)**
- 46-65 years	**40 (7.3)**	**- Emotional stability**	**28.00 (8.00)**
			28.08 (5.57)
		- Intellect/ Imagination	**35.00 (5.00)**
			35.29 (3.56)
Marital status	**N (%)**	**BDI score**	**10.00 (11.00)**
- Single	**266 (48.3)**		**11.63 (8.30)**
- Married	**245 (44.5)**	**BITE score**	**14.00 (12.00)**
- Divorced	**31 (5.6)**		**15.38 (7.70)**
- Widowed	**9 (1.6)**	**- Severity**	**3.00 (3.00)**
			3.71 (1.58)
Educational level	**N (%)**	**OCI-R score**	**41.00 (18.00)**
- High school	**65 (11.8)**		**41.27 (12.08)**
diploma	**88 (16.0)**	**- Washing**	**5.00 (4.00)**
- IEK-TEE degree	**258 (46.8)**		**5.95 (2.94)**
- TEI-AEI degree	**140 (25.4)**	**- Checking**	**6.00 (4.00)**
- Master's degree			**7.03 (3.01)**
Ethnicity	**N (%)**	**- Ordering**	**8.00 (5.00)**
- Greek	**545 (99.3)**		**8.26 (3.18)**
- Other	**4 (0.7)**	**- Obsessing**	**7.00 (5.00)**
			7.54 (3.28)
Weight satisfaction	**N (%)**	**- Hoarding**	**7.00 (5.00)**
- Not at all	**176 (31.9)**		**7.54 (3.11)**
- Relatively	**274 (49.7)**	**- Neutralizing**	**4.00 (3.00)**
- absolutely	**101 (18.3)**		**4.95 (2.32)**
ED History	**N (%)**	**EAT score**	**55.00 (11.00)**
- Yes	**94 (17.1)**		**55.00 (8.73)**
- No	**457 (82.9)**	**- Dieting**	**16.00 (5.00)**
			16.12 (3.77)
		- Food preoccupation	**24.00 (10.00)**
			22.97 (7.08)
		- Important others	**17.00 (3.00)**
			15.91 (2.78)
BMI	**22.73 (5.79)**	**EHQ score**	
	24.04 (4.79)	**- Feelings**	**11.00 (5.00)**
			10.77 (3.06)
		- Knowledge	**10.00 (5.00)**
			10.60 (3.23)
		- Problems	**15.00 (5.00)**
			16.25 (4.71)

IQR: Interquartile Range; SD: Standard Deviation; ED: Eating Disorder; BMI: Body Mass Index; IPIP: International Personality Item Pool; BDI: Beck's Depression Inventory; BITE: Bulimic Investigatory Test, Edinburg; OCI-R: Obsessive Compulsive Disorder – Revised, EAT: Eating Attitudes Test; EHQ: Eating Habits Questionnaire

IPIP evaluation, agreeableness and intellect/imagination were positively correlated with the EHQ 'Feelings' subscale, and each personality trait was positively correlated with the 'Knowledge' subscale ($p<0.05$), with all correlations being weak ($r=0.09$-0.15) None of the IPIP's subcategories was correlated with 'Problems' subscale. The BDI-II scale was positively correlated with the EHQ 'Feelings' and 'Problems' subscale, but weakly ($r=0.12$-0.15), and negatively correlated with the 'Knowledge' subscale ($p=0.004$, $p<0.0001$ and $p=0.001$ respectively). The OCI-R total score positively correlated with the EHQ 'Feelings' and 'Problems' subscale ($p=0.005$ and $p<0.0001$ respectively) in a weak manner ($r=0.02$-0.17). The 'Symptom' subscale of the BITE questionnaire positively correlated with the EHQ 'Feelings' and 'Problems' subscale, but negatively with the 'Knowledge' subscale ($p<0.0001$), while the 'Severity' subscale of the BITE questionnaire positively correlated with the EHQ 'Feelings' subscale, but negatively with the 'Knowledge' subscale ($p=0.001$ and $p<0.0001$ respectively; $r=-0.19$-0.26). The EAT total score and subscales negatively correlated with all three EHQ subscales ($p<0.05$) except

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Table 2. Rotated factor loadings of the principal components analysis (PCA) for 21 items (N= 551)

Items	EHQ Knowledge	EHQ Problems	EHQ Feelings
1. I am more informed than others about healthy eating	0.52		
3. The way my food is prepared is important in my diet	0.721		
5. My eating habits are superior to others	0.761		
11. My diet is better than other people's diets	0.736		
21. I prepare food in the most healthful way	0.667		
2. I turn down social offers that involve eating unhealthy food.		0.348	
4. I follow a diet with many rules		0.387	
6. I am distracted by thoughts of eating healthily		0.574	
7. I only eat what my diet allows		0.351	
8. My healthy eating is a significant source of stress in my relationships		0.693	
10.My diet affects the type of employment I would take		0.554	
13. In the past year, friends or family members have told me that I'm overly concerned with eating healthily.		0.559	
14. I have difficulty finding restaurants that serve the foods I eat		0.597	
16. Few foods are healthy for me to eat		0.618	
17. I go out less since I began eating healthily		0.582	
18. I spend more than three hours a day thinking about healthy food.		0.685	
20. I follow a health-food diet rigidly		0.415	
9. I have made efforts to eat more healthily over time			0.694
12. I feel in control when I eat healthily			0.817
15. Eating the way I do gives me a sense of satisfaction			0.842
19. I feel great when I eat healthily			0.814
Eigenvalues	6.269	2.121	1.731
% of Variance	29.854	10.099	8.244
Croncbach's alpha	0.796	0.82	0.817

EHQ: Eating Habits Questionnaire

Table 3. Correlations (Spearman's rho) between EHQ subscales.

	EHQ Feelings	EHQ Knowledge	EHQ Problems
EHQ Feelings	1	0.443**	0.477**
EHQ Knowledge		1	0.521**
EHQ Problems			1

Table 4. Descriptive characteristics of the three subscales of EHQ (1st and 2nd measurement) and test-retest reliability.

Subscale	Items	Range	Mean	SD	Minimum	Maximum	p-value
EHQ Feelings 1st time	4	1-16	10.77	3.06	4	16	0.388
EHQ Feelings 2nd time			11.2	3.49	4	16	
EHQ Knowledge 1st time	5	1-20	10.6	3.23	5	20	0.596
EHQ Knowledge 2nd time			12	3.88	5	20	
EHQ Problems 1st time	12	1-48	16.25	4.71	12	39	0.666
EHQ Problems 2nd time			15.5	4.38	12	31	

SD: standard deviation, Wilcoxon, $p<0.05$; EHQ: Eating Habits Questionnaire

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Table 5. Associations between EHQ subscales and other study measurements.

Study measurements	Categories	EHQ Feelings	EHQ Knowledge	EHQ Problems
Sex Median (IQR) Mean (SD)	Males	11.00 (4.00) 10.09 (3.27)	11.00 (5.00) 10.35 (2.95)	15.00 (7.00) 16.37 (4.85)
	Females	11.00 (5.00) 10.83 (3.04)	10.00 (5.00) 10.62 (3.25)	15.00 (5.00) 16.23 (4.70)
	p-value	0.194	0.597	0.898
Education level Median (IQR) Mean (SD)	High School	11.00 (5.50) 10.00 (3.53)	11.00 (7.00) 9.40 (3.64)	23.00 (13.00) 19.80 (6.76)
	Lyceum	9.00 (5.00) 9.80 (3.25)	10.00 (4.00) 10.02 (3.10)	14.00 (3.00) 15.35 (3.82)
	Vocational Training	11.50 (5.00) 10.82 (3.15)	9.50 (4.00)* 9.77 (3.00)	15.00 (4.75) 15.78 (4.34)
	Tertiary Education	11.00 (4.00) 10.88 (2.89)	11.00 (5.00) 10.79 (3.16)	15.00 (5.00) 16.45 (4.80)
	MSc/PhD	11.00 (4.75) 10.99 (3.16)	11.00 (4.00)* 11.06 (3.42)	15.00 (5.00) 16.42 (4.98)
	p-value	0.116	0.016	0.254
Marital status Median (IQR) Mean (SD)	Single	11.00 (4.25) 10.96 (3.00)	10.00 (4.00) 10.36 (3.18)	15.00 (4.00) 16.09 (4.43)
	Married	11.00 (5.00) 10.46 (3.03)	10.00 (4.50) 10.79 (3.21)	15.00 (4.00)* 16.13 (4.93)
	Divorced	12.00 (5.00) 11.06 (3.64)	11.00 (6.00) 10.94 (3.41)	17.00 (7.00) 17.32 (4.60)
	Widowed	13.00 (5.50) 12.56 (2.83)	11.00 (7.50) 11.44 (4.12)	18.00 10.00)* 20.22 (5.31)
	p-value	0.075	0.351	0.015
IPIP Spearman rho p-value	Extraversion	0.062 0.145	0.086* 0.043	0.019 0.664
	Agreeableness	0.108* 0.012	0.093* 0.03	0.059 0.168
	Conscientiousness	0.069 0.107	0.181** 0	0.081 0.057
	Emotional stability	-0.05 0.241	0.093* 0.029	-0.009 0.828
	Intellect/ Imagination	0.095* 0.025	0.150* 0	0.051 0.236
BDI-II Spearman rho p-value	Total	0.122** 0.004	-0.135** 0.001	0.149** 0
OCI-R Spearman rho p-value	Total	0.121** 0.005	-0.057 0.185	0.156** 0
	Washing	0.019* 0.01	0.032 0.459	0.165** 0
	Checking	0.051 0.228	-0.04 0.347	0.08 0.059
	Ordering	0.068 0.11	-0.013 0.766	0.091* 0.033
	Obsessing	0.111** 0.009	-0.119** 0.005	0.108* 0.011
	Hoarding	0.072 0.09	-0.046 0.279	0.083 0.051
	Neutralizing	0.023 0.591	-0.046 0.28	0.101* 0.018
BITE Spearman rho p-value	Symptom	0.262** 0	-0.193** 0	0.175** 0
	Severity	0.148** 0.001	-0.204** 0	0.064 0.132
EAT Spearman rho p-value	Total	-0.512** 0	-0.283** 0	-0.514** 0
	Dieting	-0.354** 0	-0.524** 0	-0.442** 0
	Food preoccupation	-0.448** 0	-0.063 0.141	-0.365** 0
	Important others	0.039 0.365	-0.116** 0.007	-0.100* 0.019
BMI Spearman rho p-value		0.132 0.486	-0.319 0.085	0.112 0.557

Mann-Whitney, Kruskal Wallis, One way ANOVA, Spearman's rho, p<0.05; IPIP: International Personality Item Pool; BDI: Beck's Depression Inventory; BITE: Bulimic Investigatory Test, Edinburg; OCI-R: Obsessive Compulsive Disorder – Revised, EAT: Eating Attitudes Test; EHQ: Eating Habits Questionnaire; BMI: Body Mass Index

for the 'Important others' subscale with the EHQs' 'Feelings' subscale and 'Food preoccupation' subscale with the EHQ knowledge subscale. These correlations were weak to moderate (r = -0.10 to -0.52).

Discussion

The aim of this study was to translate and validate the Eating Habits Questionnaire in the Greek language, and investigate its psychometric properties in adults of the general population.

EFA results supported the 3-factor structure, and the 21-item version of EHQ showed satisfactory goodness-of-fit, in line with the original survey, and other validation efforts. The internal consistency, which was evaluated with Cronbach's alpha coefficient, was found satisfying, with subscale alphas varying from 0.80 to 0.82, within the range (0.75-0.90) of other validation attempts (Mohamed Halim *et al.*, 2020). The high correlation coefficient value signified the convergence of the same construct. Most EHQ items demonstrated an acceptable performance, displaying good item-total correlation coefficients, implying that all items were important components of the construct.

Criterion validity was determined by the strength of correlation between the EHQ subscales and other constructs related to orthorexia nervosa. Consistently with the original study, the subscales correlated more strongly with the questionnaires regarding eating pathology (BITE, EAT-13) than with those of obsessive-compulsive and depressive symptomatology (OCI-R, BDI-II), and personality traits (IPIP). The 'Problems' subscale showed no significant correlations with personality traits, very weak correlations with OCD and depressive symptomatology, and very weak to moderate correlations with eating pathology. However, the original study showed a positive correlation of the 'Problems' subscale with the EAT-26 scores, while this study demonstrated an up to moderate negative correlation with all the variables of the Greek version of EAT-13. This finding was supportive of other research outcomes; a study which evaluated the prevalence of ON in young adults (18-23 years of age) found a significant negative correlation between EAT-40 and ORTO-15 scores (Sanlier *et al.*, 2016). The 'Knowledge' subscale was related with personality traits and depression, contradicting the original study. However, most of the parameters evaluating abnormal eating behavior, were in line with the original study's findings. Very weak correlations were found between the 'Feelings' subscale and OCD and depression evaluation, partially contradicting the original study, which reported no correlation. However, the agreement was profound regarding the correlations with eating pathology constructs. The relationship between ON and eating pathology is presumed to be mediated by obsessive and compulsive characteristics. The fact that ON was not strongly related to OCD symptomatology contradicts existing findings which have shown that orthorexic eating and obsessive-compulsive disorder are significantly correlated, irrespectively of the severity of the disorder (Yılmaz *et al.*, 2020). This discrepancy between severity of OCD symptoms and ON, highlights the fact that ON is leaning more towards eating disorders that the OCD spectrum, verifying the stronger relation of EHQ with eating behaviour instruments found in this study.

The results showed no significant sex differences, which are in line with the current literature, though some reported discrepancies seem to depend on the measuring tool used (Strahler, 2019). Like other research, the results of this study showed that BMI was not significantly correlated with neither of the three EHQ subscales. Regarding BMI and ON dimensions, several studies indicate no significant association with BMI (Ferreira and Coimbra, 2021), or sex (Oberle *et al.*, 2018).

A strength of the present study was the use of a large population sample and the assessment of test-retest reliability of the questionnaire. Nevertheless, this study bears certain limitations. There may be some bias due to the self-report nature of the data, in the context of the inherent trend for socially desirable responses. This falls within all responses and mainly the weight and height measurements for the compute of body mass index. Besides, the voluntary participation in the research could lead to selection bias, as subjects were not invited to participate following the standard randomization method.

To conclude, our findings support that the Greek version of 21-item Eating Habits Questionnaire demonstrates good psychometric properties. Its internal consistency, test-retest reliability and criterion validity were sufficient. Orthorexia nervosa is an emerging health issue, and thereby, the validated Greek version of EHQ could be utilized as a useful tool for application in research and clinical practice. Future research should focus on clinical groups, specifically on individuals with eating or OCD spectrum disorders and adolescent populations which are considered to be at risk.

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Key Points

- When healthy eating becomes obsessive, psychological disturbances may emerge
- The overwhelming preoccupation with health eating may lead to orthorexia nervosa
- The Eating Habits Questionnaire assesses orthorexic characteristics
- The Greek version of the Eating Habits Questionnaire demonstrated good psychometric properties in adults.

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Protein.Spotlight - 244

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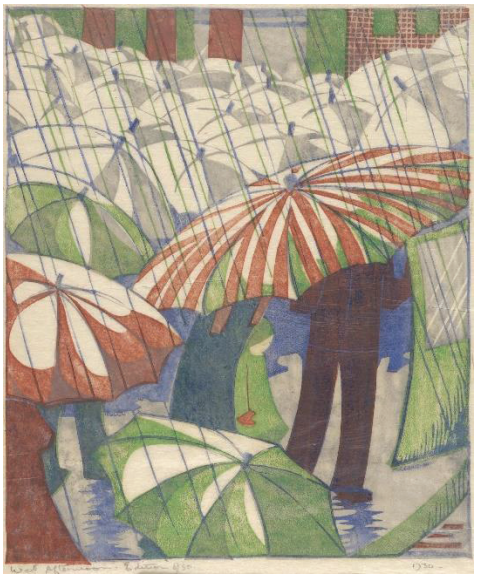
for the sake of sap

Vivienne Baillie Gerritsen

When rain is pelting down and you have no protection, the umbrellas carried by other pedestrians suddenly become attractive. The thought of making one yours might even occur to you. In the same vein, a child who is being hit with a stick by another child might decide to grab the stick and hit their assailant with it in return. Taking possession of a means of defence that does not initially belong to you is not only a time-saver but also a sure way of dealing appropriately with the enemy, since it has been used just for that. The same kind of commerce exists between all sorts of organisms, though in a far more subtle manner. Many plants produce toxic compounds to fend off insects that feed off their sap. Recently, scientists discovered that a herbivorous species of whitefly – the sweet potato whitefly *Bemisia tabaci* – seems to have acquired an enzyme of plant origin. The enzyme in question modifies specific toxins – namely phenolic glycosides or PGs – so as to render them harmless. These particular toxins are secreted by plants precisely to ward off herbivorous insects. By acquiring the plant enzyme, *B.tabaci* has also acquired the means to detoxify PGs and therefore take advantage of plants that produce them. And the enzyme is? Phenolic glucoside malonyltransferase 1 (MAT1), that we shall call BtPGMT1.



Wet Afternoon, 1930

Ethel Spowers (1890-1947)

An enzyme of plant origin in an insect? How can that happen? Can insects acquire proteins from other organisms the way you would pick a bag of crisps off a shop shelf? No. It all has to do with genetics and time – a process known as 'horizontal gene transfer'. More often than not, genes are transferred down generations. That is, offspring inherit a mixture of genes from their biological parents; this is called 'vertical gene transfer'. Sometimes, though, genetic matter can be relayed from one organism to another, even though they are not related. This frequently occurs between bacteria for example, or between viruses and plants. But genetic transfer between two eukaryotes, such as between insects and plants, is something scientists have rarely had the chance to observe – if at all.

Plants and insects have been living together on this planet long before humans were even a blip on evolution's radar. For the best part of 400 million years, they have been fine-tuning their genomes to live side by side without either of them losing too much ground. In a way, it is a constant race to arms. One will find ways to bypass the enemy's defence while the other sharpens its knives. This is what seems to have happened between the sweet potato whitefly *Bemisia tabaci* and the plants it feeds off. Based on phylogenetic analysis and comparing key domains of the enzyme BtPGMT1 in *B.tabaci* with that of hundreds of plant genomes, it is likely that *B.tabaci* stole the phenolic glucoside malonyltransferase gene, MAT1, from a plant that

grew about 86 million years ago. It was a slick move to make since it enabled the whitefly to persist on over 600 different species of toxic plants.

Insects are harmful to plants in two ways. First, they feed on their sap – which is what carries water and nutrients to every part of the plant – usually leaving in their wake the all too familiar honeydew on the plants' leaves. Second, they invariably inject pathogens such as viruses into the plants. In response, plants have designed strategies to keep insects off them, one of which is the synthesis of phenolic glycosides, or PGs. PGs are a combination of one sugar (glycone) group with one functional non-sugar (aglycone) group. As secondary metabolites, they are not involved in the plant's normal growth and development but, depending on their functional group, are used to ward off noxious organisms or sometimes even to lure them – as plant pigment flavonoids cunningly evolved to attract insects for pollination for example.

Certain PGs are toxic to insects in that they can strongly affect their growth, development and behaviour – although it is not known how exactly. While feeding off plant sap, insects will inadvertently ingest PGs that end up in their gut cells where they will exert their toxicity. Surprisingly, PGs can also be toxic to plants! This is where BtPGMT1 comes in. This particular enzyme catalyzes the transfer of a malonyl group from malonyl coenzyme A to PGs. Such a transfer, or malonylation, confers various roles to PGs, one of which is detoxification. In plants, PGs detoxified in this way are stored in vacuoles until the toxic form is activated – presumably by de-manolylation – and released upon insect invasion. When *B.tabaci* attacks a plant, the plant reacts by secreting toxic PGs into its sap, which end up in the insect's gut cells. There, they are detoxified by the insect's personal copy of BtPGMT1.

It is the detoxifying action of the plant's BtPGMT1 that will have seduced – understandably so – *B.tabaci* so many millions of years ago. If it could acquire a copy, then it would be able to neutralise the PGs it ingests and feed off the host plant unperturbed. None of this happened with clear intention on the insect's behalf naturally. As for all similar genetic events, chance played the biggest part and adaptation did the rest. Today, plant PGs are little threat to *B.tabaci*, tomorrow its host plants will have developed yet another means of defence. Such is the driving force of evolution. In the meantime, *B.tabaci* still causes damage to crops worldwide – by feeding off their phloem while injecting pathogens, all of which causes huge economical loss. Now that scientists know more about BtPGMT1 and where it originally came from, it should help to develop ways of fighting off *B.tabaci* – perhaps by tampering with BtPGMT1's gene to silence it. Thus, in a way, playing the same game and taking back what never really belonged to the whitefly in the first place.

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luck of the draw

Vivienne Baillie Gerritsen

When something gets uncomfortably close to you – in whichever way it may be – you will seek to fend it off. By walking away, choosing to ignore it, using physical force or, if it is a person, perhaps verbal abuse. In the same vein, across all kingdoms, organisms have developed a multi-faceted system to fight off the more invisible world of microbial infection: the immune response. The immune response is the arsenal an organism has at its disposal to neutralise invading entities such as viruses or bacteria for instance – and the more complex the organism the more intricate the system seems to be. Despite this, the types of armament provided are only really of two sorts: cells or molecules. Any immune response to infection is an unfathomable combination of both; there being many different kinds of immune cells and myriads of immune molecules. Sometimes, too, genetic inheritance can further fine-tune an individual's reaction to a given infection. In this light, researchers discovered that human individuals who have acquired a specific isoform of a certain protein, known as 2'-5'-oligoadenylate synthetase 1 or OAS1, seem to be less prone to developing the severe form of COVID-19.

Compulsory vaccination drive in New Jersey *ca.* 1880s

National Library of Medicine, USA

The immune response, and its many ramifications, evolved hundreds of millions of years ago with the advent of multicellular organisms. It was a case of letting similar cells cohabitate while preventing the intrusion of outsiders. In short, it was a way of distinguishing the 'self' from the 'non-self'. Nature has been chiseling and refining the immune system ever since but scientists only began to understand the molecular nature of its many components during the last century.

Though it may seem trivial and hardly worth mentioning today, the fact that pathogens – named microbes – could be the cause of certain diseases was only established in the 19th century by the German microbiologist Robert Koch. Now we know that an infection caused by microbes triggers off an immune response that is both cellular and molecular (humoral). We have immune reservoirs – such as our tonsils, our spleen, our liver or our bone marrow – and our blood and lymph ensure that the products are distributed throughout our body.

Surprisingly, although the knowledge we have on the intricacies of the immune reaction is really quite recent, the fact that you can inoculate someone with a disease, in the hope that they will develop some form of future resistance to it, is hundreds of years old. As an example, long before anything of the sort happened in Europe, people in China, Africa and India were being variolated (inoculated) with the smallpox virus – *Variola virus* – to induce immunity. Though many developed a violent reaction and infected others, when an epidemic broke out, the mortality rate in the population was far lower than if no variolation had been performed at all.

The procedure arrived in England in the 18th century. At about the same time, it became apparent that dairy farmers, or indeed their milkmaids, seemed to be preserved from smallpox outbreaks. Little by little, the farmers realised that cow udders infected with cowpox

could in turn infect humans. Cowpox happens to be similar to smallpox, only it is a milder form. Unknowingly, the act of milking cows was actually protecting milkmaids against smallpox. In the 1890s, the English physician Edward Jenner decided to test the hypothesis by presenting the cowpox virus to his gardener's son. Six weeks later, the physician presented the boy with the smallpox virus – who developed no symptoms. Despite this, variolation in Great Britain was only banned from medical practice 40 years later and replaced by Jenner's 'vaccines' – the term given by the doctor himself, meaning 'from a cow'.

Viruses are not organisms and unable to multiply without the help of hosts. This is the basis of infection. Once a virus has entered an organism, it recognizes specific host cells which it will infect. The virus will use the cell's resources to replicate its genome – sometimes in specialized organelles known as replicative organelles (ORs) – and synthesize all it needs to form its progeny, or virions. An infected cell can release up to hundreds of thousands of virions – each of which, like its parent, will infect another host cell. It is not difficult to grasp, then, that in order to halt such wild replication, the host has to react fast. One reaction is to produce small cell-signaling molecules known as cytokines that will trigger the expression of all sorts of genes involved in the immune response.

Once such cytokine is known as interferon. It is the most powerful antiviral cytokine and, rapidly deployed after infection, it activates, among other proteins, the synthesis of the 2'-5'-oligoadenylate synthetase (or OAS) family. This particular family of enzymes is able to sense foreign nucleic acid in the cell – such as viral RNA. Binding to the nucleic acid leads OAS to synthesize 2'-5' oligoadenylates which go on to activate an endoribonuclease (RNase L) that will degrade the viral RNA. In this way, the synthesis of viral proteins is prevented and the early spread of the virus kept at bay. As the now infamous COVID-19 continues to spread around the globe, scientists have had time to observe its mechanics more closely and they made an intriguing discovery: a particular isoform of the OAS family – OAS1 – seems to be involved in saving patients from developing a severe form of COVID-19.

How? OAS1 exists in a short and a long form. The long form, known as p46 and thought to be of Neanderthal provenance, is less common. It has a tail at its C-terminal that encodes for a prenylation signal – a signal believed to facilitate the attachment of proteins to cellular membranes, among which ORs. When severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects cells, it generates ORs. The viral double-stranded RNAs are lodged within the OR membranes and are immediately, and specifically, sensed by OAS1. Once docked, OAS1 synthesizes 2'-5' oligoadenylates that go on to activate a downstream RNase L that degrades the viral RNA – thus checking viral replication. It seems, therefore, that prenylated OAS1 is specifically targeted to the ORs of SARS-CoV-2, and patients with this particular isoform are less prone to developing complications due to COVID-19 because they can fight it off better.

Naturally, it is very likely that OAS1 is only one of many proteins whose synthesis is stimulated by interferon and it is therefore probably not alone in preventing an acute form of COVID-19. What is more, members of the OAS family are upregulated in the presence of other viruses, as well as in certain autoimmune diseases. Now we know that the long or the short form of OAS1 can behave differently upon SARS-CoV-2 infection, it may be that members of the OAS family behave differently in other disorders too, making them useful biomarkers at different stages of a disease thus prompting development-related therapy. Certainly, scientists have reached a very attractive hypothesis: a gene we might have inherited from the Neanderthals and which may protect many of us from developing a potent form of COVID-19.

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the colour red

Vivienne Baillie Gerritsen

Plants cannot walk. Unable to drift down to the local café, attend this evening's book launch or gate-crash a party, flowers have had to resort to other ways of connecting. True, their roots may wander and branches may wave, but really what appears above ground level is pretty moored. Yet that is where their reproductive organs are, which need to meet so that the plant's pollen can be fertilized. This is achieved indirectly by using animal pollinators – whose attention, however, needs to be grabbed. Nectar fulfils this role wonderfully. A sweet liquid secreted by flowers, nectar is concocted to tempt insects or vertebrates whose bodies, as they feed off it, may inadvertently pick up pollen in one flower and deposit it, in all innocence, on another flower's stigma. So as not to be missed, a little like waving a flag, a flower's nectar may occasionally be brightly coloured: yellow, deep purple, blue, green, red or even black. In this light, the striking red nectar of *Nesocodon mauritianus*, a blue flower endemic to the island of Mauritius, seems to have evolved to attract a day gecko and is synthesized thanks to the close collaboration of three enzymes: Nec1, Nec2 and Nec3.

Heather Angel / Natural Visions

Courtesy of the artist

It is estimated that an astounding 90% of flowering plants depend on animals to reproduce. This has been – and continues to be – an endless source of co-evolution down the years, as plants adapt to lure pollinators while pollinators adapt to find food. With time, plants have developed many different ways of attracting pollinators by using a complex combination of flower size, colour, shape, scent, taste and even landing platforms known as spurs! Mainly composed of sugars, nectar constitutes a rich food source for those who find it. It is secreted by a gland, called a nectary, which is either situated within the flower or external to it. Nectaries tucked inside flowers are intended for pollinators. Those found outside flowers seem to have a defensive role, attracting animals that lap up the nectar while also feeding off the odd plant-eating insect that happens to be present. In both cases, nectar can be seen as a form of reward where the animals are given something in return for their involuntary services.

Nectar is a rich sweet liquid mainly composed of sucrose, glucose and fructose in varying proportions. It may also contain certain antimicrobial or antifungal proteins – as in tobacco plants for instance – to defend the reproductive organs from pathogens. Nectar can also be delivered with a taste, a scent and sometimes too a colour. Each of these properties may change depending on the flower's reproductive maturity – since progeny is really what it is all about. As an example, some flowers give their nectar a bitter taste until they are ready to reproduce while others produce a bright colour only when they are ready. This is the case of *Nesocodon mauritianus*, the blue Mauritius bellflower, whose nectar goes from a 'not ready' yellow, to an 'almost there' orange and a final 'go for it' bright red. Not only does the bright red express sexual maturity but it is also a sure way of attracting a pollinator's attention – while still sending out an 'honest signal' since it also means there is something nourishing to eat.

What gives the nectar of *N.mauritianus* its colour? How does it turn red? Most floral nectars are not

coloured but usually as clear as water. The scarlet red nectar of the blue Mauritius bellflower is caused by a pigment that has been called 'nesocodin'. This bright red pigment stems from an initial aurone found in the nectary; a type of flavonoid that provides a yellow colour to flowers. It takes about 24 hours for the yellow to turn red, and this is achieved thanks to a very tight collaboration between three enzymes: a carbonic anhydrase (Nec1), a flavoenzyme (Nec3) and a ferritin-like catalase (Nec2). In a nutshell, Nec3 oxidizes sinapyl alcohol – exported from the nectary – to produce the pigment precursor sinapaldehyde. The red pigment nesocodin is then spontaneously formed by the condensation of sinapaldehyde and proline. However, these two reactions can only take place if there is an increase in the environment's pH, which is brought about by Nec1 thanks to the presence of bicarbonate, itself probably provided by nectary respiration. Finally, Nec2 protects nesocodin from degradation by breaking down the toxic by-product (hydrogen peroxide) which is released by Nec3.

Currently, of an estimated total of about 370,000 species of flowering plants worldwide, barely 70 produce coloured nectar – ranging from yellow, amber, red, brown, green, blue and black. Instinctively, one would suppose that coloured nectar attracts pollinators – and perhaps very specific pollinators – far better than nectar which has no colour or is just a honey-like pale yellow. Against its bright-blue petals, the scarlet red nectar in *N.mauritianus* flowers certainly strikes as being quite conspicuous, much like waving a flag for attention. Why choose red, you may wonder. Several arguments have been put forward. First, *N.mauritianus* grows on the island's steep cliffs – which makes it a plant difficult to reach. A bright-coloured nectar would act like a beacon. *N.mauritianus* may, also, have specifically evolved to produce red-coloured nectar to attract a particular vertebrate: the endemic *Phelsuma* day gecko that lives on the cliffs, has excellent colour vision and loves bright colours, namely red. This said, geckos may not be the only pollinators – the bellflower's red nectar may well attract certain birds too.

Painting nectar a scarlet red would send out a rather clear signal to any passing forager. But how can we be sure that geckos, or birds for that matter, see the same bright red we see in *N.mauritianus*? We cannot. But what has been observed is that *Phelsuma* day geckos not only prefer red nectar to yellow nectar but they also ignore red nectar which has been put into red flowers. It is therefore the contrast between the two colours which is important. And the bigger the contrast, the more attractive – or noticeable – the nectar must be. Of equal interest is the nectar of a flower found thousands of miles away from *N.mauritianus* and endemic to the mountainous Andean region of South America: *Jaltomata herrerae*. The nectar of this flower, visited by humming birds, is also red and the enzymes which produce the pigment are analogous (but not similar) to those used by *N.mauritianus*. This would imply that their genes have evolved independently and gained related functions to produce the same kind of nectar – in both instances very distinguishable in extreme topographies. In a way, it is a little vexing to realise that the astonishing array of colours, scents and shapes flowers display are never developed for the pleasure of human senses but for those of their pollinators – but we can thank Nature for the extent of her imagination.

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a heated legacy

Vivienne Baillie Gerritsen

Stress. We know what it feels like. Though we may be the only living organism to have turned it into a fertile and imaginative piece of conversation, every single living species on this planet is prone to stress and its effects. It comes in many forms – heat, cold, hunger, overwork, noise, pressure, weight, toxicity – and gives rise to an array of symptoms such as migraine, fatigue, weariness, depression, indigestion, eczema, insomnia, and this is only an anthropocentric list! Sloths, birds, grapes or butterflies may not be accustomed to work overload or headaches but they do suffer from heat, drought or deforestation for example, as do so many species on earth. To guarantee their survival and hence reproduction, organisms have developed mechanisms to challenge stress. Some plants for example, are equipped with sensors that detect heat shock thereby setting off pathways that will not only protect the plant but also compel it to flower faster under the strain. Some plants also seem to acquire the capacity to remember heat and react faster to it when it occurs again, or even to transmit this memory to their progeny. How? Thanks to the action of at least two proteins: HSFA2 and HTT5.

Carl Friedrich Philipp von Martius

German botanist and explorer (1794-1868)

Heat stress is brought about by temperature changes, themselves induced by natural causes or the now sadly infamous global warming due to increasing human activity since the industrial revolution. There have been episodes of global warming in the past – such as the Paleocene-Eocene Thermal Maximum (PETM) which was possibly caused by volcanic activity which generated extreme changes in the Earth's carbon cycle. This occurred about 55 million years ago when the Earth's temperatures increased by 5 to 8 °C over a period of about 200,000 years causing the mass extinction of certain species while others fled to different parts of the planet. The global warming we are experiencing now may sound less extreme but it is frequently compared to the PETM because of the mass of carbon that is being flung continuously into our atmosphere and the amount of carbon the Earth's oceans and forests are having to deal with – but cannot.

Greenhouse gases – namely CO_2 and methane – are to blame for global warming. While the sun is able to shine through them, the heat the sun generates on the Earth's surface has difficulty finding its way out again. Consequently, the Earth's atmosphere is slowly getting warmer, creating myriads of climatic disparities we hear about on a daily basis. It took a long time for humans to admit that their activity was responsible for warming up the planet disastrously. The 'greenhouse effect' had already been proposed in the 1820s and an article said to

have first appeared in the journal Popular Mechanics (New York) in March 1912 clearly described the "*furnaces of the world [...] burning about 2,000,000,000 tons of coal a year*" creating a "*blanket for the earth [...] to raise its temperature*". Yet theories of the like were met with general skepticism until the 1980s – and it took a further 40 years for the United Nations to state officially that climate change caused by humans is indisputable.

Every single living species on this Earth – in water, air and on land – is having to deal with climate change. Over the course of this year, which part of the world did not have too much rain, not enough rain, too much heat or too much cold, and witnessed the devastation of many of its crops? Plants react strongly to dramatic increases in heat, and every level of a plant cell is affected. Cell membrane fluidity is impaired, membrane proteins are damaged, enzymes are denatured, pathways are flawed, gene expression is altered, chloroplasts become non-functional, photosynthesis grinds to a halt. In short, heat creates a dramatic imbalance that – unless foreseen or checked – ends up killing them. Who has not seen garden flowers wither under stifling heat? The ability for plants to respond to heat stress existed long before present-day global warming. However, the phenomenon's persistence is gradually modifying how plants relate to their environment. What is more, plants seem to be able to remember the occurrence of a former heat shock and respond to a second one faster – a phenomenon known as plant thermomemory, or thermal acquired tolerance. It seems too, that some plants can transmit this memory to their offspring, something known as transgenerational thermomemory.

Heat shock is defined as a temperature which is higher than the temperature plants are used to, and which causes irreversible harm to their growth and productivity. How do plants deal with it? Plant cells are equipped with thermosensors that sense changes in environmental heat and relay the information to heat shock transcription factors (HSFs). In turn, HSFs – which have the capacity to switch genes on or off – promote the production of a selection of heat shock proteins (HSPs), each of which works towards protecting plant cells from the effects of heat, such as checking protein denaturation or aggregation for example. Plants that have already been through a period of heat stress seem to deal with heat better than plants that have not, as though they had acquired a sort of tolerance to it. This is not a scoop, but what has come as a surprise is that this acquired tolerance sometimes seems to be passed down the generations. How is this explained?

Epigenetics may be the answer, that is to say the inheritance of modifications in regions which flank genes. Changes in these regions bring about changes in gene transcription. Like a switch: either a gene is transcribed, or it is not. To keep things simple, if thermomemory is inherited, perhaps it is because certain genes influenced by former heat stress are simply kept 'switched on' so to speak. Transgenerational thermomemory is a complicated affair, which – like all pathways – involves the activation of a team of interacting proteins. So let us, for simplicity's sake, just hover over the very broad lines. As always, several proteins are part of the process but we will only mention two: 1) HSFA2 which responds directly to a shift in heat and 2), Heat Induced Tas1 Target 5, or HTT5, which is directly involved in sparking off early flowering. In short, when a plant is subjected to heat stress, HSFA2 is activated, and promotes the expression of certain proteins which go on to trigger the production of HTT5. HTT5 causes the plant to flower earlier than it would have normally. Meanwhile, the expression of HSFA2 is upregulated by the proteins it upregulates, creating a positive feedback loop considered to be fundamental for the establishment of thermomemory and for its transmission down the generations.

Early flowering would then be a plant's answer to a shift in heat – although it is done at a cost, since it also reduces the plant's resistance to disease. Adaptation comes with compromises. Thermomemory, and its inheritance, is an exciting field for researchers. Would it be possible to engineer crops to acquire thermomemory? If so, would the plants pass it down equally to their offspring? A godsend… There is still so much to understand, however. The basics of what is going on may have been unravelled but no one has managed to put their fingers on the heat sensors, for instance, nor understand how the transmission of thermomemory actually occurs in detail. It is, nonetheless, another wonderful example of how Nature responds to changes. Heat stress? Nature will find a way around it, adapting and evolving as she always does. There are tipping points though – beyond which the task of adapting may become too hard. Let us hope that we humans will respond in time to help and do our bit.

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