VOLUME
3
ISSUE 1
(2022)

NATURAL SCIENCES

The Journal of the Institute of Advanced Research

The University for Innovation

Editor-in-Chief

Professor Rao Bhamidimarri
President
Institute of Advanced Research

Volume Editor

Prof. Anand K. Tiwari
Institute of Advanced Research

NATURAL SCIENCES

The Journal of the Institute of Advanced Research

The journal of Natural Sciences is a multi-disciplinary research journal, published by the Institute of Advanced Research, *The University for Innovation*, Gandhinagar, Gujarat, India.

The purpose of the journal is to provide a platform for disseminating novel and innovative research and development to help address the global grand challenges that we confront.

The journal publishes peer reviewed scientific and technical articles in all disciplines encompassing natural sciences and those at the interfaces of natural sciences and other disciplines.

The journal can be accessed for free at
https://www.iar.ac.in/naturalsciences

Information for Authors

https://www.iar.ac.in/journal/authorinformation

Editorial Office

The Journal of Natural Sciences
Institute of Advanced Research
Koba Institutional Area
Gandhinagar – 382 426
India

NATURAL SCIENCES

The Journal of the Institute of Advanced Research

Foreword

Research in universities and research institutions has traditionally been discipline based. Therefore, research in institutions is organized to promote research in respective disciplines. To address the challenges we face today, such as climate change, long-term health, and frequent common infectious diseases, we need to cross-fertilize ideas from many disciplines such as science, engineering, social sciences, and humanities.

Multidisciplinary approach is traditionally directed at problem solving, for example in engineering. However, knowledge enhancement at the interfaces of disciplines is critically important in order to find novel solutions to increasingly complex problems. Multidisciplinary research is very much in vogue. Multidisciplinary research requires inputs from a variety of individual disciplines operating in a culture of collaborative exploration across the discipline boundaries. More and more institutions recognize the need for facilitating multi-disciplinary research and development and promoting multidisciplinary teams and research.

Recent decades have seen exciting multidisciplinary research and novel solutions being found based on new knowledge.

I am pleased to introduce the first issue of Natural Sciences, a new journal dedicated to provide a venue for dissemination of multidisciplinary research and development. While there are several other platforms for multidisciplinary and interdisciplinary research, this publication is intended for research with direct impact on emerging challenges.

Professor Rao Bhamidimarri

Editor-in-Chief

NATURAL SCIENCES

The Journal of the Institute of Advanced Research

Research Article

Differential characteristics of accessory genome aids in evolutionary divergence in enterococcal species

Parva Mandeep Dhruv[a*], Shreeya Himanshubhai Mehta[a*], Hetasmi Ketankumar Bhavsar[a*], Mayuri Arvindbhai Mahala[a*], Shruti Soni[a*], Hetvi Saurin Shah[a*], and Utpal Bakshi[a#]

[a]*Bioinformatics Laboratory, School of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institutional Area, Gandhinagar 382426, India*

** Equal contribution*

 # Corresponding Author: utpal.bakshi@iar.ac.in

Abstract

From the time of its origin (i.e. ~500 million years ago), the organisms of the genus Enterococcus are highly adapted to live in the harsh environment of the earth. As far as current understanding of their ecological niche distribution, they are mainly natural inhabitants of gastrointestinal tracts of a wide variety of animals, from insects to man. The explicit evolutionary adaptation of enterococcal species, especially *E. faecalis* and *E. faecium*, in the human gut, has made them the leading cause of multidrug-resistant hospital acquired infections nowadays. In this study, we have reported that two species of enterococci that are dominant in the human gut and play vital roles in hospital-acquired infections, *E. faecalis*, and *E. faecium*, harbor distinct sets of accessory genomes. Characteristically, the *E. faecalis* accessory genomes are significantly overabundant in metabolism-related Cluster of Orthologous Groups (COGs) of proteins, although the carbohydrate metabolism COGs are higher in *E. faecium* accessory genomes. The unusual distribution of carbohydrate metabolism-specific COGs highlight the unique abilities of *E. faecium* to metabolize sugars from the plant origin. Accessory genomes of *E. faecalis* are found to have more enriched Intrinsically Disordered Proteins (IDPs) distribution which is interacting with each other through protein-protein interaction (PPI) pathways. Most of the accessory gene products of *E.faecalis* involved in PPI pathways are cell wall anchor domain-containing proteins, which are significantly found to contain evolutionary conserved stretches of Intrinsically Disordered Regions (IDRs) in their sequences. Contrastingly in *E. faecalis*, signatures of positive selection were found in a few of these protein sequences that signify mutational adaptation of these important classes of conserved IDRs in *E. faecalis* accessory genomes.Thus, in a nutshell, the current study addresses the importance of accessory genomes in the evolutionary divergences of enterococcal species and provides plausible evolutionary significant roles of groups of proteins on enterococcal adaptation to its distinct habitat.

Keywords: pangenome, intrinsically disordered proteins, enterococci

1. Introduction

1.1 Background

The genus Enterococcus contains ecologically diverse species including facultative anaerobic, non-spore-forming and catalase-negative (Foulquie Moreno et al., 2006; Van Tyne and Gilmore, 2014) group of bacteria. The habitat distributions of enterococci are found to be diverse and are associated with humans, other mammals, reptiles, amphibians, birds, and insects (Santagati et al., 2012). However, to date, enterococcal species of only humans and domestic animals have been studied thoroughly. The major interest in studying enterococci has mainly been generated due to their potential to cause diseases by acquiring drug-resistant properties. The pathogenic characteristics have mainly been observed in two species of enterococci – *Enterococcus faecalis* and *Enterococcus faecium*, and research on them is largely concerned with the development of antibiotic resistance in them (Plotnikava et al., 2017; Markwart et al., 2019; Pöntinen et al., 2021).

Next-generation sequencing technologies have given the opportunity to carry out large-scale comparative studies on bacterial genomes to establish patterns of behavior and evolutionary relationships (Land et al., 2015). Since the introduction of the concept of pan-genome (Tettelin et al., 2005), numerous studies have reported that the gene content of a genome and its organization can be affected during organisms' adaptation to a specific niche and the major driving factors for adaptation are horizontal gene transfer (HGT), genome rearrangements, and gene loss (Altermann, 2012; Emamalipour et al., 2020). These phenomena have been shown to affect the genomic integrity of organisms. For example, in the bacterial family Vibrionaceae, Kahlke et al., 2012 showed that unique core genes appear more often in groups of isolates with a common ancestor (monophyletic) and in the particular case of *Vibrio cholerae*, play a fundamental role in its adaptation to the ecological niche. Likewise, the same study indicated that in genophyletic groups of isolates (with no closest common ancestor), core genes are mainly the result of HGT. In *Pseudomonas putida*,the presence of genomic islands carrying genes related to its specific lifestyle suggests HGT as the major driving force of its adaptation to a certain niche (Wu et al., 2011).

In enterococci, comparative pan-genomic analysis has mostly been performed in two prominent enterococcal species in humans - *E. faecalis* and *E. faecium*, revealing their unique genomic characteristics, specifically the influence of recombination in the evolution of accessory genomes of *E. faecium* (de Been et al., 2013; Kim and Marco, 2014) and pathogenic island acquisition in the genomes of *E. faecalis* (Bakshi et al., 2016). Recently, a pan-genomic study of enterococcal phyla has been carried out by (Zhong et al., 2017); however, this study, which primarily focused on the host origin of enterococci and the distribution of common families of antibiotic resistance genes, hardly addressed the issue of wide divergences in the accessory gene repertoire of different species of

enterococci. Recent reports hint towards significantly different characteristics of the non-conserved parts of the enterococci genomes, viz., distribution of mobile genomic elements (Santagati et al., 2012), recombination blocks (de Been et al., 2013), pathogenic islands (Li and Wang, 2021), and IDP cassettes (Bakshi et al., 2016), etc. Nevertheless, current understanding on enterococcal pangenome did not throw any light on differential properties in the non-conserved genomes and their role in evolutionary divergence among different species of enterococci.

1.2 Diverse habitat distribution of enterococcal species

Despite having disputes on its taxonomic allocation, present-day enterococci have been classified into mainly 17 different species (Santagati et al., 2012). Although they evolved over time as a member of a broad group of GI tract commensal consortia, in recent times, few of the members emerge as a leading cause of hospital-acquired infections of the bloodstream, urinary tract, surgical wounds, and other body sites (Khan et al., 2015; Brinkwirth et al., 2021). Among the seventeen species of enterococci, two species viz., *E. faecalis* and *E. faecium* are mainly responsible for hospital acquired infections with a proportion of 80-90% for *E. faecalis* and 10-20% for *E. faecium* (Mascini and Bonten, 2005, Shridhar and Dhanashree, 2019). Other species of enterococci are typically associated with the intestine of humans and domestic animals. When enterococcal species are found outside the gut,they are either typical indicator of fecal pollution or in the case of the human body, a possible pathogen (Byappanahalli et al., 2012; Li et al., 2021).

Of the 17 main species of enterococci, complete genome sequence information is available for 10 species (Table 1). The species of enterococci can be distinguished by various biochemical and genomic characteristics, e.g., glycopeptides resistance is associated with two distinct species – i) van-c1 ligase specific *E. gallinarum* and ii) van-c2/3 ligase specific *E. casseliflavus* (Reid et al., 2001). Two species of phylogenetically related enterococci, viz., *E. durans* and *E. hirae* are infrequently isolated from humans and more frequently from the guts of domestic animal species (Devriese et al., 2002). Infections by these two species in humans are apparently rare. Another rarely found species of enterococci in the human gut is *E. mundtii*. Recent studies have shown that *E. mundtii* is one of the prominent microbiota in some leaf insects (Chen et al., 2016). Their highly active metabolic role in these species of insects hint towards their importance in host biology. Three species of enterococci with newly sequenced genomes form aberrant branches in enterococcal phylogeny, viz. *E. silesiacus*, *E. thailandicus*, and *E. wangshanyuanii*. These species are isolated from widely distributed sites, viz. insect gut (*E. silesiacus)* to bovine feces (*E. thailandicus* and *E. wangshanyuanii).* Distinct phenotypic features and DNA-DNA hybridization experiments confirm their species status in enterococcal phylogeny (Lebreton et al., 2014).

1.3 Genomic diversity of enterococci in host: role of non-conserved genome

One of the major limitations of comparative genome-based studies of enterococcal species is the lower number of sequenced genomes, compared to other bacterial species. Genome sequencing data of only two species of enterococci are widely available: *E. faecalis* and *E. faecium.*

The sequencing of *E. faecalis* V583 genome was started in the late 1990's and completed in 2002. This genome sequence, though contained a major portion of PAI, mobile genetic elements (MGE), and plasmids carrying antibiotic-resistance determinants lacked the crucial antibiotic-resistant genes esp and cyl, as a 17-kb DNA fragment carrying these genes had been excised from the PAI itself (Paulsen et al., 2003). The sequencing of the V583 genome appeared to provide new insight into enterococcal genomes, their genetic makeup, and biology. Unfortunately, the number of sequenced complete genomes remained low in enterococci until very recently, when a number of complete genomesequences have been added to the database mainly either as a part of the Human Microbiome Project (http://www.hmpdacc.org/) or as a part of multi-national collaboration with the Broad Institute (Cambridge, MA). Genome analysis of the *E. faecalis* revealed a number of significant features. The strain *E. faecalis* 62 was isolated in a healthy Norwegian infant in 2006 and belonged to CC6, which had never been associated with nosocomial infections. In this genome, the presence of genomic islands (GIs) carrying genes involved in lactose and other carbohydrate metabolisms instead of virulence determinants, emphasized its adaptation to its commensal existence (Solheim et al., 2011). In 2007, partial genome analysis of the commercial probiotic strain *E. faecalis* Symbioflor was made. This strain did not possess any virulence determinants, and for this reason, was proposed as a probiotic, but no information was available due to the absence of sequence data for this strain (Domann et al., 2007).

In the case of *E. faecium*, different genome sequencing projects in different ecological niches yield three basic observations: (i) hospital-associated isolates accumulate genomic differences related to antibiotic resistance and colonization genes; (ii) strains belonging to the same CC, e.g, CC17, have a large difference in their accessory gene content; and (iii) the pan-genome analysis of *E. faecium* indicated that the total available gene pool within these species is essentially unlimited, depending onthe ecological niches that these species can colonize (Van Schaik et al., 2010).

All these published genome-based studies of enterococci have contributed to the understanding of genomic diversity, especially in *E. faecalis* and *E. faecium*, confirming the affirmation of specific sub-populations associated with humans which possess large differences in their accessory genes, which in turn play key roles in the evolution of enterococcal species.

1.4 Intrinsically disordered proteins in enterococcal genome

With the growing number of experimental data on protein structure determination in bacterial genomes, it is evident that a large number of proteins, under physiological conditions, do not possess

a well-defined 3D structure (Uversky, 2013). The intrinsic disorder has been reported in the bacterial genome both at a sequence as well as structural level and is characterized by dynamic ensembles of structures instead of one single structure. Some well-known functions of disordered proteins include molecular recognition and assembly (as encountered in signaling pathways), protein modification (e.g., phosphorylation, acetylation, methylation, etc.), and entropic chain activities (e.g., linkers, springs, and spacers) (Van Der Lee et al., 2014). The molecular recognition and assembly functions of disordered proteins are typically a direct consequence of disorder-to-order transitions that can readily occur in flexible, disordered regions of proteins. The functional diversity provided by disordered regions has been conjectured to largely complement the functions of ordered proteins.

Outer membrane proteins of several pathogenic microbes are found to be intrinsically disordered (Tusnády et al., 2015). Recently in *E. faecium*, a group of cell wall anchor proteins that play a key role in bacterial colonization and virulence is found to be intrinsically disordered (Galloway-Peña et al., 2015). In *E. faecalis* virulent strains, a genomic cassette coding for four functional proteins, which itself are a part of pathogenic island, are found to be intrinsically disordered (Bakshi et al., 2016). Although these studies identify a number of intrinsically disordered proteins in enterococci, to date no detailed genome-wide analysis of the distribution of IDPs in enterococcal genome has been performed. Such analysis may indicate the functional significance of these classes of proteins in enterococcal evolution.

With the above understanding, the main objectives of this current study include: (a) Comparative genome analysis of major enterococcal species in human microbiota to decipher their structural and functional characteristics; (b) correlating the pan-genomic distribution of gene families with the evolution of enterococcal species; (c) investigate the importance of IDPs and their subsequent characterization in enterococcal genomes.

2. Materials and methods

2.*1 Sequence data collection*

Complete genome sequences of 59 enterococcal species available during the start of the analysis were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/). The two most abundant species of enterococcal phyla in the dataset are *E. faecium* (30 genomes) and *E. faecalis* (15 genomes), with 14 other species of enterococci. The host specificity of the isolates was confirmed by PATRIC database (Wattam et al., 2013). Most of the members of *E. faecalis* and *E. faecium* were isolated from humans, whereas other species were mostly associated with the gut of other vertebrates (Table 1).

2.2 Pan-genomics and phylogenetic analysis

Pan-genome analyses of 59 enterococcal species were carried out in BPGA pipeline with the default 50-50 criteria (identity-coverage) (Chaudhari et al., 2016). Binary gene matrix data file was further processed to detect species specific gene family enrichment and construct pan-genome phylogeny

using PAST (Hammer et al., 2001). Species in binary data files are clustered by single-linkage Euclidean clustering method and the statistical robustness of the tree was checked by 100 bootstrap replicates. For comparative analysis, 16S and core genome phylogeny of the enterococcal species were also constructed in the dataset. 16S genes of the complete genomes of enterococci were extracted from NCBI; for core genome phylogeny, the enterococcal core gene set was aligned and refined as described previously (Bakshi et al., 2016). Finally, with a set of 561 core gene families, a phylogenetic tree was constructed using the maximum likelihood method with RAxML8, with 100 bootstraps replicate, and using the PROTGAMMA algorithm (Stamatakis, 2014).

Table 1: Details of enterococcal species under study

No.	Name of the Organism	Isolated From	Accession	Size (Mb)	GC %	CDS	Protein
1	*E. casseliflavus EC20*	Animal	PRJNA32935	3.4	42.8	3285	3148
2	*E. durans KLDS6.0933*	Animal	PRJNA291957	3.1	37.8	2989	2537
3	*E. durans KLDS6.0930*	Animal	PRJNA291946	3.1	37.8	2987	2521
4	*E. durans BDGP3*	Animal	PRJNA397225	3.0	38.0	2868	2614
5	*E. faecalis V583*	Human	PRJNA70	3.4	37.4	3412	3264
6	*E. faecalis OG1RF*	Human	PRJNA20843	2.7	37.8	2710	2602
7	*E. faecalis 62*	Human	PRJNA61185	3.1	37.4	3157	307575
8	*E. faecalis D32*	Animal	PRJNA169860	3.1	37.4	3174	2973
9	*E. faecalis str. Symbioflor 1*	Human	PRJEB648	2.8	37.7	2885	2733
10	*E. faecalis DENG1*	Human	PRJNA187445	3.0	37.5	3050	2881
11	*E. faecalis ATCC 29212*	Human	PRJNA244550	3.0	37.4	3128	2922
12	*E. faecalis LD33*	Animal	PRJNA316564	2.8	37.6	2867	2695
13	*E. faecalis KB1*	Animal	PRJNA317592	3.0	37.2	3014	2815
14	*E. faecalis L9*	Animal	PRJNA352597	2.7	37.7	2706	2578
15	*E. faecalis L12*	Animal	PRJNA352597	2.7	37.8	2660	2543
16	*E. faecalis CLB21560*	Human	PRJNA368724	3.2	37.1	3404	3211
17	*E. faecalis sorialis*	Human	PRJNA318633	3.1	37.2	3059	2886
18	*E. faecalis FDAARGOS 338*	Human	PRJNA231221	2.9	37.6	2939	2572
19	*E. faecalis W11*	Human	PRJDB5023	2.7	37.7	2699	2577

20	*E. faecium DO*	Human	PRJNA30627	3.1	37.9	3209	3114
21	*E. faecium Aus0004*	Human	PRJNA86649	3.0	38.3	3118	2919
22	*E. faecium NRRL B-2354*	Animal	PRJNA74725	2.8	37.9	2920	2719
23	*E. faecium Aus0085*	Human	PRJNA193299	3.2	38.0	3381	3004
24	*E. faecium T110*	Human	PRJNA207757	2.7	38.5	2764	2551
25	*E. faecium 64/3*	Human	PRJNA293793	2.6	38.2	2631	2441
26	*E. faecium UW7606x64/3 TC1*	Human	PRJNA299371	2.8	38.2	2821	2626
27	*E. faecium 6E6*	Human	PRJNA308959	3.4	37.6	3558	3294
28	*E. faecium UW8175*	Human	PRJNA275194	2.9	38.0	2966	2746
29	*E. faecium E39*	Human	PRJNA281247	3.1	37.8	3204	2984
30	*E. faecium ISMMS VRE 1*	Human	PRJNA292786	3.3	37.7	3384	3104
31	*E. faecium ISMMS VRE 7*	Human	PRJNA292801	3.2	37.7	3350	3076
32	*E. faecium ISMMS VRE 11*	Human	PRJNA292807	3.1	37.7	3245	2910
33	*E. faecium E745*	Human	PRJNA295268	3.2	37.7	3279	3037
34	*E. faecium E1*	Human	PRJNA353757	3.2	37.7	3332	3104
35	*E. faecium VRE001*	Human	PRJNA353653	3.2	37.8	3351	3075
36	*E. faecium ISMMS VRE 12*	Human	PRJNA292809	3.1	37.8	3235	2999
37	*E. faecium ISMMS VRE 9*	Human	PRJNA292804	3.1	37.8	3231	2994
38	*E. faecium 2014-VREF-41*	Human	PRJNA358851	3.3	37.6	3381	3115
39	*E. faecium 2014-VREF-114*	Human	PRJNA358851	3.3	37.5	3386	3067
40	*E. faecium 2014-VREF-268*	Human	PRJNA358851	3.4	37.6	3526	3246
41	*E. faecium 2014-VREF-63*	Human	PRJNA358851	3.2	37.6	3335	3074
42	*E. faecium K60-39*	Human	PRJNA407052	3.1	37.7	3164	2879
43	*E. faecium A 020709 82*	Human	PRJNA344739	3.1	37.8	3162	2936
44	*E. faecium E240*	Human	PRJNA349966	3.1	37.8	3286	3028
45	*E. faecium E243*	Human	PRJNA349966	3.1	37.8	3288	3028
46	*E. faecium 16-346*	Human	PRJNA389415	2.7	38.1	2825	2416

47	*E. faecium EFE10021*	Human	PRJEB12395	2.6	38.1	2647	2465
48	*E. faecium aus00233*	Human	PRJEB14733	3.3	37.8	3382	3137
49	*E. faecium ATCC 700221*	Human	PRJNA311738	3.2	37.8	3145	2725
50	*E. gallinarum FDAARGOS 163*	Animal	PRJNA231221	3.8	42.3	3638	3395
51	*E. gallinarum FDAARGOS 375*	Human	PRJNA231221	3.5	42.2	3494	3283
52	*E. hirae ATCC 9790*	Animal	PRJNA49385	2.9	36.9	2752	2481
53	*E. hirae R17*	Animal	PRJNA319460	3.0	37.0	2685	2512
54	*E. hirae FDAARGOS 234*	Animal	PRJNA231221	2.8	37.0	2593	2359
55	*E. mundtii QU 25*	Animal	PRJDB868	3.4	38.3	3229	2992
56	*E. mundtii EMB156*	Animal	PRJNA337899	3.0	38.5	2867	2653
57	*E. silesiacus LMG 23085*	Animal	PRJNA226735	3.9	36.4	3627	3559
58	*E. thailandicus a523*	Animal	PRJNA400701	2.6	36.8	2499	2370
59	*E. wangshanyuanii MN05*	Animal	PRJNA388020	4.2	37.3	4197	4048

2.3 Protein functional analysis

To annotate function of enterococcal proteins, NCBI's COG category classification system was used. COGs were annotated by performing RPSBLAST of each protein sequence in the dataset against NCBI's COG database with an E-value cutoff <0.001 in WEBMGA server (Wu et al., 2011). Statistical significance of the distribution of COG categories was measured by nonparametric chi-square analysis (in a 2x2 contingency table) using STATISTICA (Version 6.0) (StatSoft, 2001).

2.4 Genome-scale Average Nucleotide Identity (ANI) calculation

To check the genomic variability of different enterococcal species, genome-wide Average Nucleotide Identity (ANI) analyses were performed using ANI calculator (Goris et al., 2007;Rodriguez and Konstantinidis, 2014). Roughly in this method, genomic similarity in genome contigsis assessed by local sequence alignment by progressively increasing the alignment window, and incorporating new contig sequence in each new turn. The analysis yields a 59x59 data matrix, which can ~~be~~ further be represented as a half-matrix because the upper and lower parts of the matrix are mere rpititions of each other.

2.5 Analysis of Intrinsically Disorder Proteins (IDPs) in enterococci

2.5.1 Identification and characterization of IDPs in enterococcal species

Genomic IDPs in the enterococcal species were identified using the DisPC pipeline (https://sourceforge.net/projects/dispc/). The pipeline first refined the protein sequences and then calculated the disordered proteins using seven distinct disorder prediction modules. The final disordered proteins were selected based on consensus prediction of IUPRED (Long) PONDR (vsl2b)- DisEMBL (Coils) method which exhibited maximum specificity and comparable sensitivity to singlepredictors in the test dataset of all disorder proteins of Disprot database (Sickmeier et al., 2006). Predicted IDPs were further characterized in the downstream analysis of DisPC pipeline. Disordered regions, which had a stretch of at least 30 consecutive disordered residues, were selected for downstream analysis.

2.5.2 Identification of protein-protein interactions

Protein-protein interaction (ppi) pathways in enterococcal genomes were determined in STRING-DB (Szklarczyk et al., 2014). Orthologous nodes (BLAST e-value <1e-5) of *E. faecalis* and *E. faecium* were clustered based on curated PPI data obtained from six different predicted interactions, viz. neighborhood, fusion, co-occurrence, text mining, co-expression, and structural homology. PPI enrichment p-values were considered to be significant if p < 1e-05. Protein functional enrichment in the predicted network was detected by similarity search of protein domains against INTERPRO database (Apweiler et al., 2001) with a false discovery rate (FDR) of < 1e- 05.

2.5.3 Identification of evolutionary selection in enterococcal protein families

The program timezone was used to characterize the evolution of gene families across enterococcal species (Chattopadhyay et al., 2013). This program executes a pipeline designed to identify genes exhibiting signatures of positive selection and recombination. It detects the presence of convergence/ hotspot mutation (i.e. independent mutation on the same amino acid position) and calculates the ratio of non-synonymous /structural (dN) and synonymous /silent (dS) substitution to infer selection through statistically defined bootstrap replication criteria. In general, dN/dS ratios below 1 are indicative of purifying selection, while dN/dS ratios above 1 are indicative of positive selection.

3. Results and discussion

3.1 Genomic heterogeneity in enterococcal species

To gain insights into the genomic architecture of the enterococcal species, a comparative genome-scale study on these bacteria was carried out. Enterococcal species vary widely both in their overall genome size and genomic GC percentages (Table 1). For example, a commensal strain of *E. faecium* 64/3 shows features of highly reduced genome with a genome size of 2.5 Mb. The number of functional genes in this organism is 2631; whereas *E. wangshanyuanii* MN05, a species of Enterococci that first isolated from fecal materials of yaks, exhibit a large genome with a genome size of 4.1 Mb and 4197 functional genes.

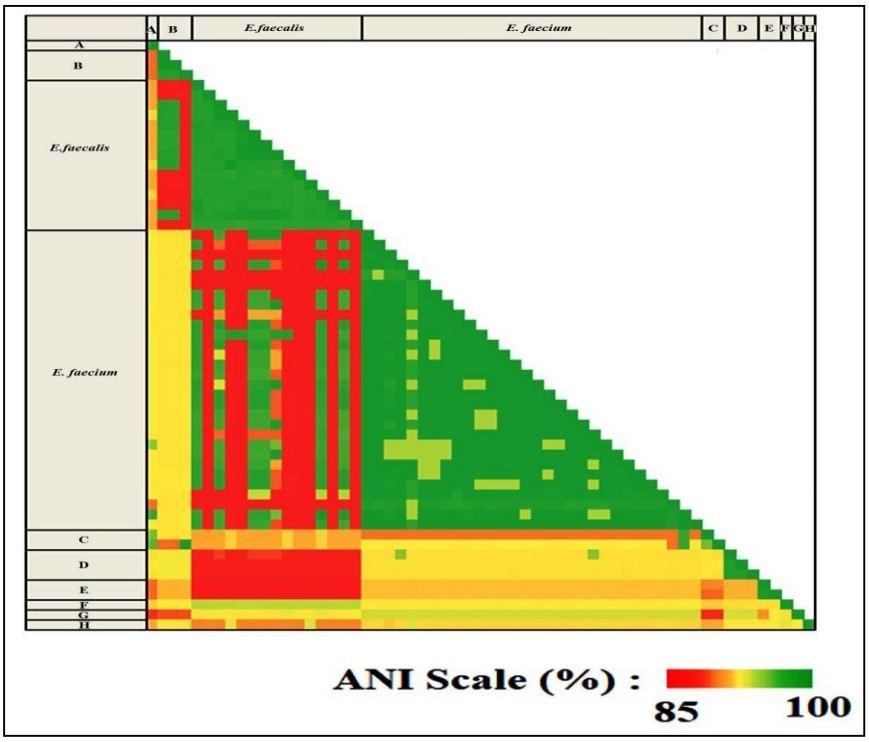


Figure 1: Genome similarity matrix of different species of enterococci

Genome-wide ANI matrix (Figure 1) shows two characteristics of genomic features in the dataset. Firstly, *E. faecalis* genomes are more homogenous than their closely related species, *E. faecium.* As evident from the matrix, genome variations among strains of *E. faecium* are much more than strains of *E. faecalis.* Literature survey has indicated that this genomic heterogeneity in *E. faecium* may be caused by recombination and HGT events, both of which are shown to be prominent features in the genome of *E. faecium* than in that of *E. faecalis* (Willems et al., 2005, Palmer et al., 2012). The second unusual feature observed from the genomic similarity matrix is the genomic relationship of *E. gallinarum* strains with *E. faecium* species group. *E. gallinarum,* a low-level vancomycin-resistant strain and the only other known enterococcal species besides *E. faecium* and *E. faecalis* to cause hospital acquired infections, is believed to diverge from *E. faecium* from evolutionary history (Palmer et al., 2012). The genomes of two strains of *E. gallinarum* in the dataset show contrasting genomic relationships with *E. faecium* group. In *E. gallinarum* FDAARGOS163, genomic similarities are assessed through Average Nucleotide Identity (ANI) percentage value wherein thelower color heat map shows the distribution of values. Prominent members of enterococcal species are *E. faecalis* and *E. faecium*, while other species represented in different alphabets in the figure are: (a) *E. casseliflavus* (b*) E. durans* (c) *E. gallinarum* (d) *E. hirae* (e) *E. mundtii* (f) *E. silesiacus* (g) *E. thailandicus* and (h) *E. wangshanyuanii.* The average genomic ANI value in *E. faecium* group is 81.3 percent while in *E. gallinarum* FDAARGOS375, it is 93.3 percent. In fact, the genome of *E. gallinarum*

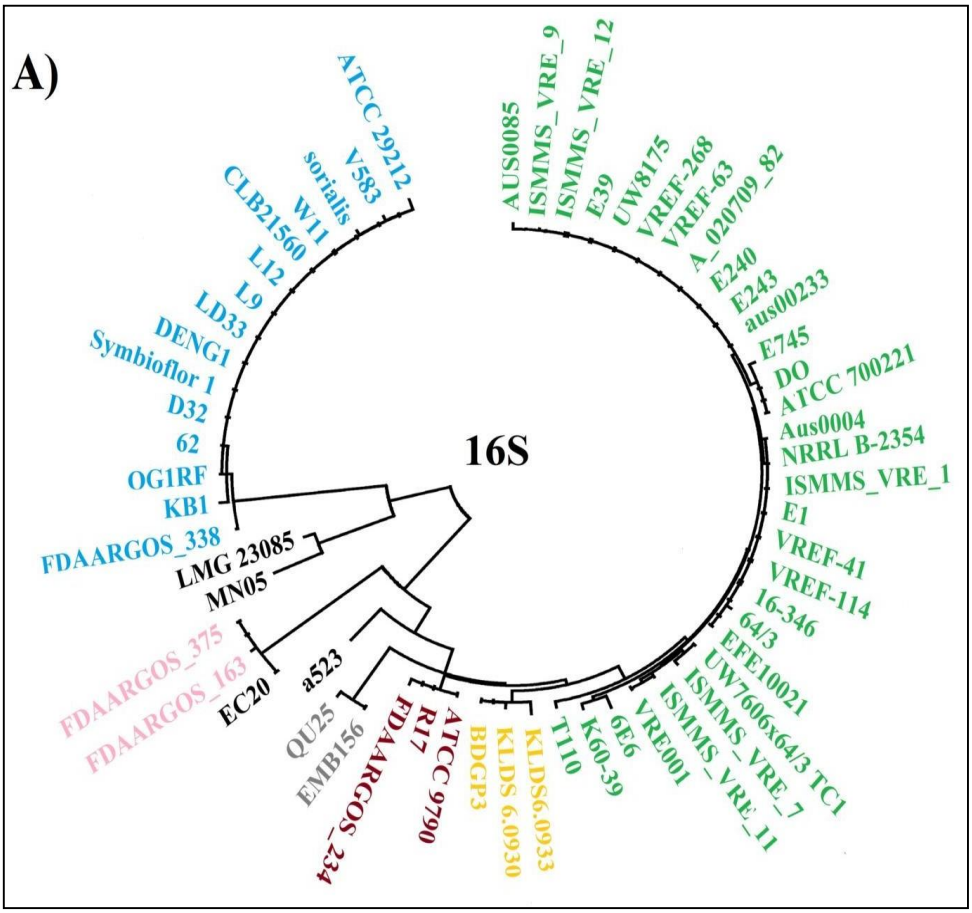
FDAARGOS163 was foundto be more similar to *E. faecalis* group than *E. faecium*. The unusual genomic divergences among *E.gallinarum* strains with respect to *E. faecium* group raise interesting questions relating to their taxonomy and evolution, the detailed analysis of that, however, is beyond the scope of this study.

Overall, the genome-wide ANI analysis helps us to take a gaze at the genomic relationship of enterococcal species which can further be explored by comparative genomics and phylogenetic study.

3.2 Pan-genomics and phylogenetic analysis of enterococci

Comparative analysis of enterococcal species revealed that enterococcal pan-genome is composed of 15478 gene families which are ~5 folds of the size of an average enterococcal genome. The core and unique genome constitute 3.6 and 29.7 percent of the pan-genome (561 and 4596 gene families, respectively), while the accessory genome contains 66.7 percent of the pan-genomic gene families (10321 genes). The small size of the conserved/core genome of enterococci harmonizes with their reduced genome features which are well documented in literature (Palmer et al., 2012). Due to a million years of co-evolution in the gut environment, species diversity in enterococci is shaped by factors such as vertical transmission, immune selection, diet, and use of antibiotics (Ley et al., 2008; Jernberg et al., 2010; Walter and Ley, 2011). Reduced genomes of enterococci have highlighted their characteristics of acquisition of many nutrients required for survival from their habitats, as opposed to carrying additional genes for biosynthesis of these nutrients (Palmer et al., 2012).

Previous phylogenetic analyses have segregated enterococcal species into different groups; some of them with respect to habitats in which these species were identified (Lebreton et al., 2014).



Two of the largest members of the enterococcal species, *E. faecium* and *E. faecali* have been clustered into different groups in phylogenetic analysis: (i) *E. faecalis*, which emerged as an important opportunistic hospital-acquiredpathogen, has been a member of the gut microbiome from the Devonian period (Gilmore et al., 2013) and (ii) *E. faecium,* which are more ubiquitous and can be found along the food chain from insects to mammals (Guzman et al., 2016).

To decipher the phylogenetic relationship of enterococcal species in the dataset, both 16S and core-genome SNP-based phylogenetic trees were constructed (Figure 2). Both of the trees are similar in structure and revealed the clade-specific structure of enterococcal species. Although 16S and core genome phylogeny segregate the major enterococcal classes, recent comparative genomic study on the core genome of two of the major clades- *E. faecium* and *E. faecalis* - has not found distinct genomic compositions among these species (Kim and Marco, 2014). Instead, a number of experimental studies, mainly through comparative genomic hybridization (CGH), have reported that global genetic diversity of enterococci is determined mainly by variation in the non-conserved/accessory genomes (Lepage et al., 2006; Van and Willems, 2010). Previous studies of genome repertoire also establish the importance of accessory genome in determining genetic diversity

in *E. faecalis* (Bakshi et al., 2016). To evaluate the genomic diversity at species level, an accessory genome phylogenetic tree from the binary pan-genome matrix was constructed (Figure 3), which represents the distribution of shared genes among different members of enterococcal phyla. The accessory genome phylogeny is congruent with 16S and core genome phylogeny of enterococci.

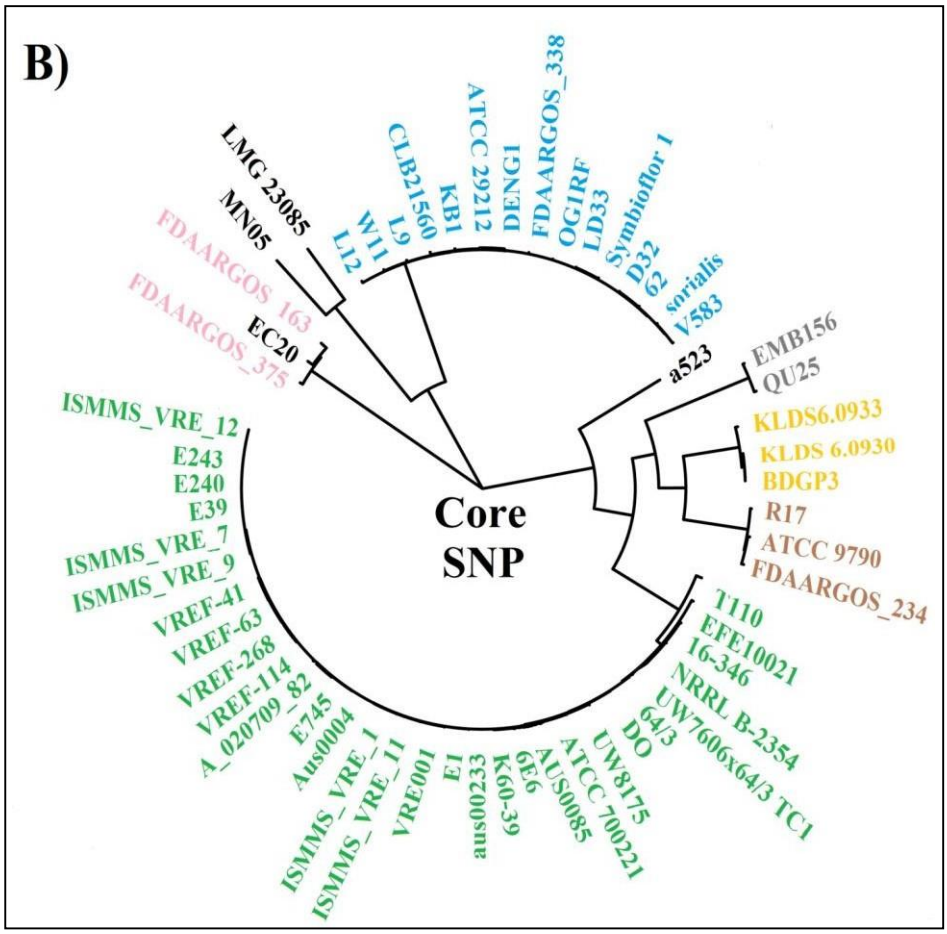


Figure 2: A) 16S and B) Core genome SNP based phylogeny of enterococcal species

Strains of different species of enterococci are highlighted in different colors: Blue – *E. faecalis*, Green – *E. faecium*, Golden – *E.durans*, Brown – *E.hirae*, Grey – *E. mundtii*, Pink- *E. gallinarum*. Enterococcal members with single species are shown in black. Both the Phylogenetic trees were constructed by RAxML8 with 100 bootstrap replicate. All the braches in both the trees have a bootstrap value >80 suggesting that accessory genome distribution differs among different members of enterococci in linewith their evolutionary divergence. One of the interesting features to note from the phylogeny is the segregation of *E. faecalis* branch from other enterococcal branches, indicating the distinct accessory genome composition of *E. faecalis*, which is supported by previous experimental evidence of accumulation of PAI and phage, HGT, and recombination signatures in *E. faecalis* genomes (Yasminet al., 2010; Werner et al., 2013; Duerkop et al., 2014).

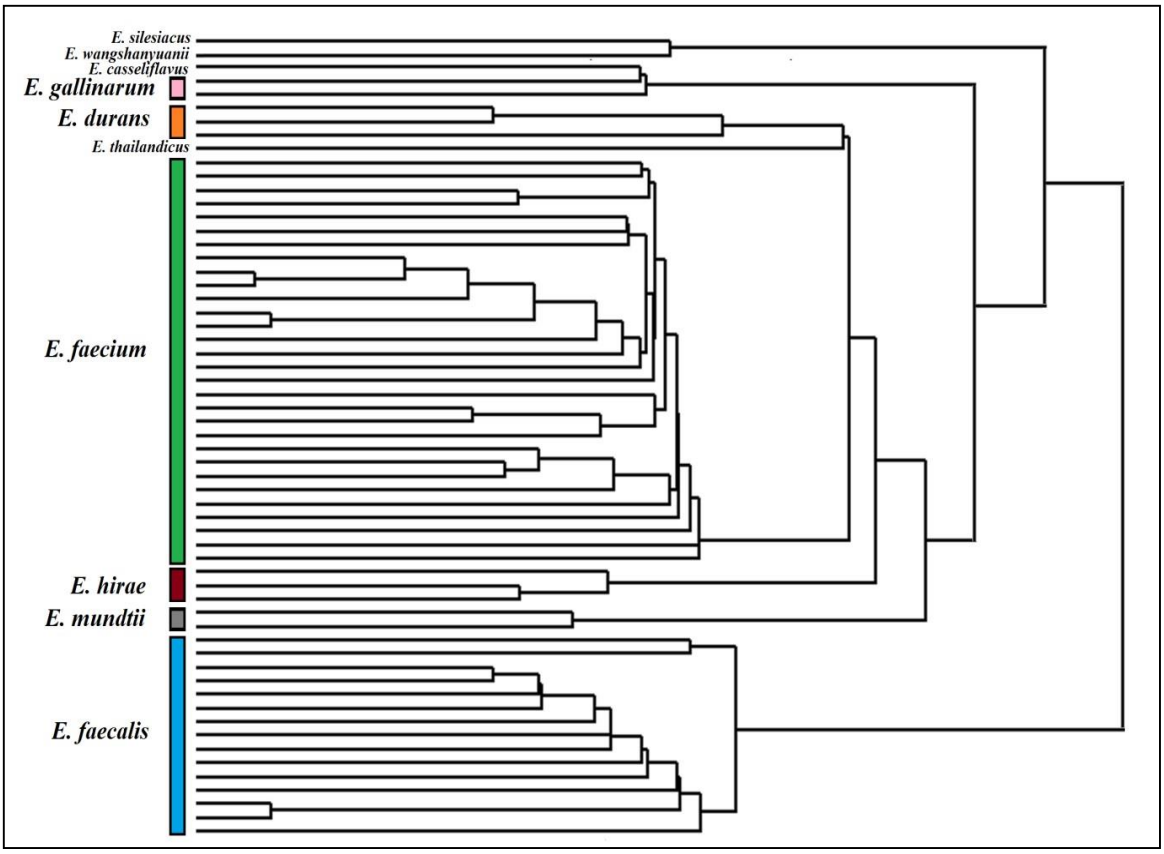


Figure 3: Accessory genome-based phylogenetic analysis of enterococcal species

Different strains of enterococcal species are highlighted in different colors. Species with more than one strain are clubbed together. The color pattern is similar to Figure 2. Enterococcal members with single species are shown in black. Phylogenetic tree was constructed by RAxML8 with 100 bootstrap replicates. All the braches in the tree have a bootstrap value >80.

3.3 Variations in accessory genomes

From the phylogenetic analysis, it is clear that pan-genomic and more specifically, accessory gene distribution play a significant role in species differentiation in enterococci. This results in good agreement with the earlier analysis of *E. faecalis* strains (Bakshi et al., 2016), where accessory genome-based phylogeny segregates the pathogenic and commensal strains more efficiently than other phylogenetic methods.

For downstream comparative analysis of accessory genomes, the dataset is restricted to two significantly abundant species of enterococci, - *E. faecalis* and *E. faecium*, as (i) in the dataset, only *E. faecalis* and *E. faecium* have sufficient number of genomes for comparative analysis and (ii) these two species of enterococci are dominant member of human gut microbiota and together they cause ~99% of enterococcal related disorders in humans.

To understand the functional variations, if any, in the accessory genomes of enterococcal species;

COG functional classification system, which is based on orthologous relationships among proteins (Tatusov et al., 2001) has been used. The accessory genomes of *E. faecalis* and *E. faecium* are separately mapped to COG database (with a PSI-BLAST criteria <1e-05) and a comparative statistical analysis using non-parametric chi-square test is performed to determine whether they have any significant difference or not.

By studying the COG categories, it is evident that there are no significant differences in the distribution of COGs in four major functional categories. *E. faecium* accessory genome has a higher abundance of 'information storage and processing' (19.88% in *E. faecium and* 17.70% in *E. faecalis*) and 'cellular processes and signaling' (19.27% in *E. faecium* and 18.05% in *E. faecalis*) specific categories, whereas accessory genomes of *E. faecalis* are more enriched in 'metabolism' related COG functions (40.93% in *E. faecalis* and 38.91% in *E. faecium*). A significant part of COGs belongto poorly characterized classes (23.23% in *E. faecalis* and 21.94% in *E. faecium*).

However, among the subcategories of the main COG functional category, there is a characteristically different pattern among metabolism-specific COGs of *E. faecalis* and *E. faecium*. Metabolism COG category has been divided into eight sub-categories, viz., Energy production and conversion (C), Carbohydrate transport and metabolism (G), Amino acid transport and metabolism (E), Nucleotide transport and metabolism (F), Coenzyme transport and metabolism (H), Lipid transport and metabolism (I), Inorganic ion transport and metabolism (P) and Secondary metabolites biosynthesis, transport and catabolism (Q). In the accessory genome of *E. faecalis*, five of the eight COG subcategories are significantly overabundant than *E. faecium* accessory genome (Table 2).

Table 2: Frequency distribution of metabolism category COGs in *E. faecalis* and *E. faecium* accessory genome

COG Category (Metabolism)	**COG ID**	**Frequency in *E. faecalis***	**Frequency in *E. faecium***	**Statistically significant (p<0.05)**
Inorganic ion transport and metabolism	P	9.15	6.30	Yes

Amino acid transport and metabolism	E	9.93	7.64	Yes
Energy production and conversion	C	4.32	2.91	Yes
Nucleotide transport and metabolism	F	2.07	0.85	Yes
Secondary metabolites biosynthesis, transport, and catabolism	Q	2.68	1.45	Yes
Coenzyme transport and metabolism	H	3.02	2.55	NA
Lipid transport and metabolism	I	1.33	1.30	NA
Carbohydrate transport and metabolism	G	8.46	15.88	Yes

Two other subcategories of COG, Coenzyme transport and metabolism (H), Lipid transport and metabolism (I), also have similar features. It is surprising to note that despite such wide divergences among frequencies of various COG categories, the overall distribution of metabolic COG in the two species is not significantly different (i.e. 40.93% COGs in *E. faecalis* and 38.91%in *E. faecium*). Further analysis revealed that a subcategory of metabolism specific COG, viz., Carbohydrate transport and metabolism (G) which is substantially over represented in *E. faecium* accessory genome are mainly responsible to increase the overall frequency of metabolism-specific COGs in *E. faecium* (Figure 4).

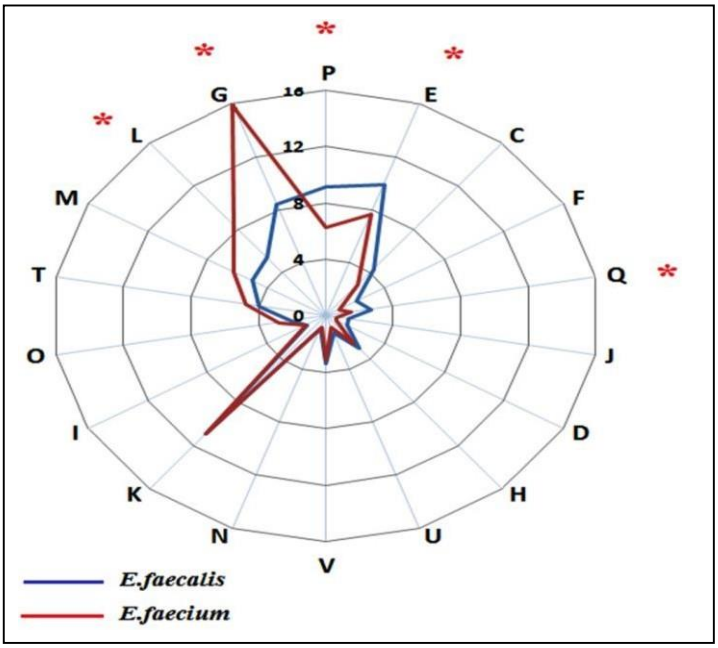


Figure 4: Frequency distribution of COG functional classes in accessory genomes of *E. faecalis* and *E. faecium*

COG categories which are significantly over and underrepresented are marked with red stars. Frequency values are shown in the radar plot. COG functional categories represented by one letter code are- J: Translation, ribosomal structure and biogenesis, K: Transcription, L: Replication, recombination and repair, D: Cell cycle control, cell division, chromosome partitioning, V: Defense mechanisms, T: Signal transduction mechanisms, M: Cell wall/ membrane/envelope biogenesis, N: Cell motility, U: Intracellular trafficking, secretion, and vesicular transport, O: Posttranslational modification, protein turnover, chaperones, C: Energy production and conversion, G: Carbohydrate transport and metabolism, E: Amino acid transport and metabolism, F: Nucleotide transport and metabolism, H: Coenzyme transport and metabolism, I: Lipid transport and metabolism, P: Inorganic ion transport and metabolism, Q: Secondary metabolites biosynthesis, transport and catabolism.

This observation of abundance of the COG category, 'G' in the *E. faecium* accessory genome is supported by a recent experimental study that revealed specifically that *E. faecium* are more enriched in carbohydrate metabolism genes than *E. faecalis* due to their ability to metabolize carbohydrate from the plant origin, which is absent in *E. faecalis* (Van Schaik et al., 2010). However, specific enrichment of only carbohydrate metabolizing genes in the accessory genomes of *E. faecium* is a novel finding.

Previous reports have identified widespread genomic heterogeneity between species (Jackson et al., 2011; Rouli et al., 2015) and even strains (Bullman et al., 2013) in the dispensable genome of various pathogenic bacteria. Heterogeneity in genomes can be represented in many ways, viz., local and global variation among GC content, genomic reorganization due to HGT events, etc. Genomic heterogeneity in enterococci is well reported in literature. These studies describe different pathogenic islands (Semedo-Lemsaddek et al., 2009), genomic cassettes (Ballering et al., 2009; Bonacina et al., 2016), and putative operons (Boyd et al., 2012; Ramsey et al., 2014) that effectively distinguish strains of both clinical and environmental origin. In an earlier study, Bakshi et al., reported wide occurrence of IDPs in the accessory genomes of E. faecalis (Bakshi et al., 2016). It is of great interest to observe whether any significant differences exist in the distribution of IDPs among the accessory genomes of two prominent enterococcal members of human microbiome - *E. faecalis* and *E. faecium*, and if such differences produce any functional divergences in these two species.

3.4 Intrinsically disorder proteins in enterococcal accessory genome

Characterizations of the IDPs in enterococcal genomes reveal many significant features related to functional and evolutionary aspects of enterococcal evolution. DisPC pipeline with stringent disorder prediction criteria through consensus approach yield 105 and 64 IDPs in *E. faecalis* and *E. faecium* accessory genome respectively. It is to be noted that this study identifies only those proteins having IDRs stretches of >30 amino acids long to be IDPs, as suggested by Dunker et al. (Dunker et al., 2000).

3.4.1 Length-dependent variation

DisPC pipeline indicates that *E. faecalis* accessory genome has higher abundance of IDPs than accessory genome of *E. faecium* (105 and 64 IDPs respectively). Further comparative analysis of the IDPs sequences indicates a significant length variation among the IDPs of two species in the accessory genome (Figure 5A). The average IDP length in *E. faecalis* is 576 amino acids with maximum and minimum values of 3088 and 101 amino acids respectively. The average IDP length in *E. faecium* is much lower, i.e. 479 amino acids with maximum and minimum values of 1179 and 101 amino acids respectively. Length distribution histogram of IDP sequences of *E. faecalis* and *E. faecium* accessory genome shows that the length of the IDP sequences is almost always much higher in *E. faecalis* with very few exceptions (Figure 5B). These larger IDPs in *E. faecalis* accessory genome could result in differential structural characterization and functional efficiency as it is well known that large IDPs are more efficient in compaction of their IDRs, which in turn modulate the function of IDPs (Uversky et al., 2012).

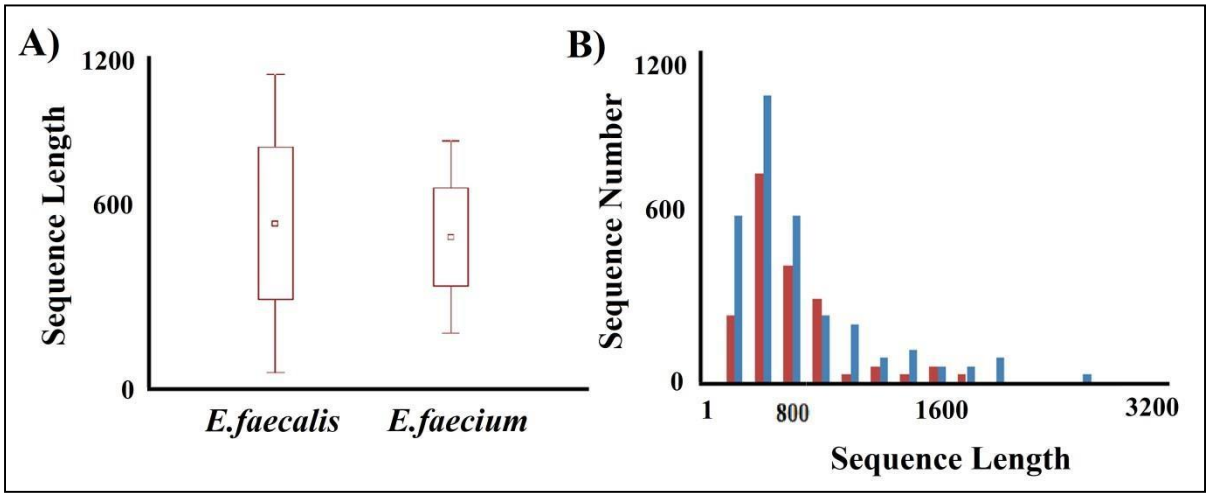


Figure 5: Length distribution of IDPs in accessory genomes of *E. faecalis* and *E. faecium*

A) The central box represents average length, the outer large box represents the standard deviation of high variation and the upper and lower end of central vertical line represents maximum and minimum amino acids length, respectively. B) Distribution of number of IDP sequences with respect to ranges of sequence length. Red and blue bars are accessory genome IDPs of *E. faecium* and *E. faecalis*, respectively.

3.4.2 Protein-protein interaction among IDP members

Protein functional analysis in the IDPs of accessory genome suggests *E. faecalis* has on average, larger IDP sequences when compared with those of *E. faecium*, which is enriched in metabolic proteins. To understand whether these groups of proteins play any role in functional divergence of these two species by interacting with the other members, a protein protein interaction (PPI) network

is constructed on STRING server (Szklarczyk et al., 2014).

Six different methods of protein interaction detection are employed, viz., neighborhood, fusion, co-occurrence, text mining, co-expression, and protein homology. *E. faecalis* specific PPI network has 67 nodes and 68 edges (p < 1e-16) which highlights that disorder proteins in *E. faecalis* are functionally connected as a group. The largest continuous interconnected sub-network in *E. faecalis* constitutes 37 nodes (Figure 6).

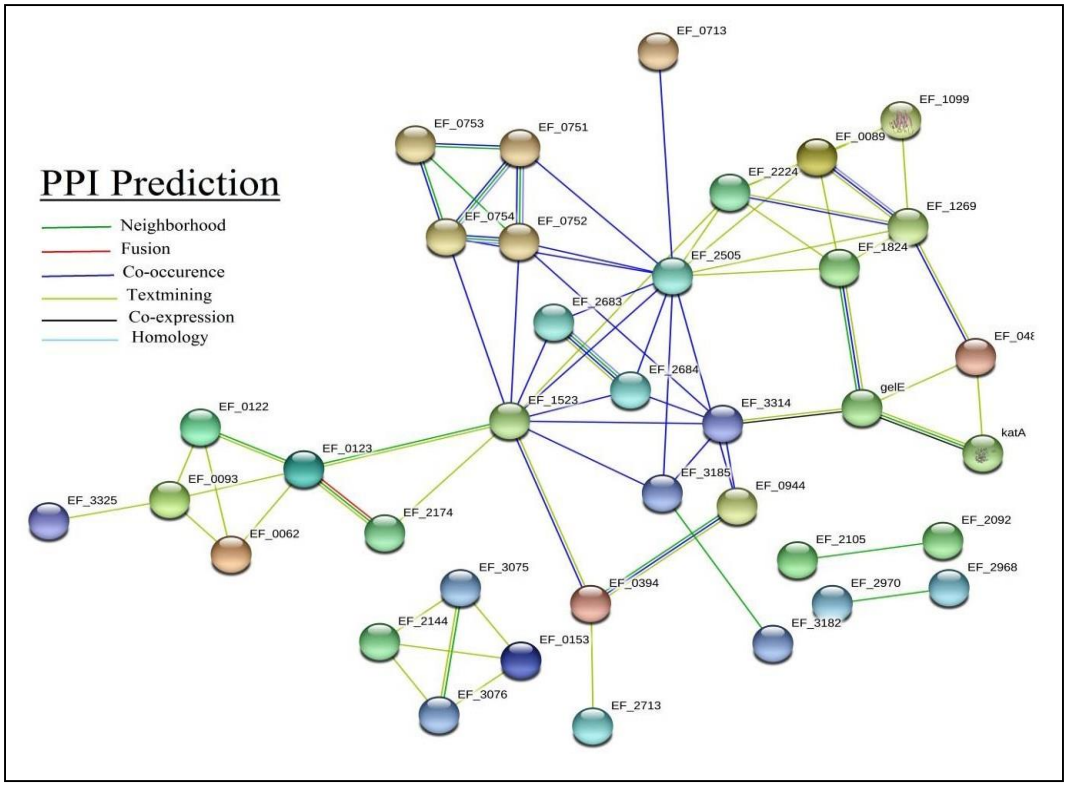


Figure 6: Prediction of Protein-Protein Interaction (PPI) among IDPs of *E. faecalis* accessory genome

Six different methods of predicted PPI are shown in the figure. PPI enrichment of the network is calculated with a p-value < 1e-05. Domain analysis of these nodes suggests that cell wall anchor proteins are significantly enriched inPPI gene set of *E. faecalis* accessory genome (Table 3).

Table 3: Functional enrichments in PPI network of *E. faecalis* accessory genome

INTERPRO Protein Domains and Features			
Pathway ID	Pathway description	Count in gene set	False discovery rate
IPR019931	LPXTG cell wall anchor domain	17	7.43E-18
IPR027994	WxL domain	12	6.38E-12
IPR019948	Gram-positive anchor	7	5.04E-06
IPR008966	Adhesion domain	4	0.000125
IPR011252	Fibrogen-binding domain	4	0.000125
IPR008456	Collagen binding domain	3	0.00506

Presence of these proteins in *E. faecalis* accessory genome suggests the possible mode of action of IDPs through protein-protein interactions and modulating signaling pathways. In the case of *E. faecium*, the PPI network has 36 nodes and 10 edges ($p < 9e-06$). There are no significantly connected large nodes in PPI and the domain analysis of the 36 nodes does not indicate any significant functional enrichment in the PPI network of *E. faecium*.

3.4.3 Evolutionary conservation and selection

Recent scientific understanding of protein intrinsic disorder reveals that a significant fraction of IDPs is evolutionary conserved across different domains of life (Ba et al., 2012; Ota and Fukuchi, 2017; Gao et al., 2021). A recent study on the evolutionary conservation of IDPs proposes that stabilizing selection in IDRs could allow quantitative phenotypes to be maintained within an optimal range while allowing tolerance of mutations or insertions and deletions, as these individually exert weak functional and selective effects (Zarin et al., 2017). Although it is highly likely that a part of IDRs are either non-functional or sites of lineage-specific evolution, at least a portion of these IDRs may be performing quantitative functions that are under stabilizing selection (Landry et al., 2014).

In this study, a comparative genomic analysis was carried out to detect any evolutionary conserved IDPs in enterococcal species. Despite having somewhat equal accessory genome content (2741 and 2567 gene families, respectively), the evolutionary conserved IDP distributions are significantly

different in *E. faecalis* and *E. faecium.* Among 105 IDPs in *E. faecalis*, 12 have been found to contain evolutionarily conserved motifs; whereas in *E. faecium*, 3 out of 64 IDPs have such regions (Table 4). The functional diversity and cellular distribution of these proteins reveal some interesting features. 10 out of 12 proteins in *E. faecalis* are annotated, 7 of which are found in the cell wall/cell membrane of *E. faecalis.* On the contrary, all 3 evolutionary conserved IDPs in *E. faecium* are cytosolic proteins.

Table 4: Evolutionary conserved IDPs in E. faecalis and E. faecium accessory genome

Protein ID	**Location**	**Function**
A) Evolutionary Conserved IDPs in *E. faecalis*		
AIL05308.1	Cell Membrane	leucine Rich Repeat family protein
AIL05646.1	Cell Membrane	membrane insertase
AIL03767.1	Cell Membrane	ABC transporter, ATP-binding protein
AIL05678.1	Cell Wall	extracellular protein
AIL03185.1	Cell Wall	cell wall surface anchor protein
ARV02399.1	Cell Wall	LPXTG-motif cell wall anchor domain protein
APE71703.1	Cell Wall	LPXTG-motif cell wall anchor domain protein
AIL04735.1	Cytosol	Catalase
AIL04689.1	Cytosol	Gelatinase
ANU73543.1	Cytosol	single-stranded DNA-binding protein
ANU73809.1	NA	hypothetical protein A4V06_12600
ANU71609.1	NA	hypothetical protein

B) Evolutionary Conserved IDPs in *E.facium*		
AFK60384.1	Cytosol	mobilization protein A (plasmid)
AQY30484.1	Cytosol	hypothetical protein B4W80_16620 (plasmid)
AFK60351.1	Cytosol	plasmid recombinase enzyme Pre (plasmid)

The cell wall/cell membrane proteins of *E. faecalis,* when mapped via KEGG database (Kanehisa and Goto, 2000) are found to be part of bacterial secretion pathway; whereas the three evolutionary conserved proteins in *E. faecium* belong to plasmid mobilization pathway. In *E. faecalis*, cell wall/membrane-derived proteins involved in secretion pathways belong to a broader class of signaling function proteins. Recent experimental studies have proposed that natural selection conserve signaling function in IDPs of human (Zarin et al., 2017). The above findings hint towards similar characteristics in bacterial IDPs. However, the conservation of plasmid function IDPs in *E. faecium* accessory genome is a novel finding and probably coupled with the genomic heterogeneity and recent genomic diversity of this species of enterococci.

In the study of human IDPs, researchers have found that though regions of orthologous IDPs are often conserved to maintain regulatory functions, there is a great amount of sequence divergence present in the regions besides IDRs (Zarin et al., 2017). If the situation is similar in bacteria, it will be interesting to check whether those variable regions confer any selection pressure on the encoded proteins. Microbial adaptive evolution prediction package TimeZone was used (Chattopadhyay et al., 2013) to determine selection footprints in the evolutionary conserved IDPs.

From the total of 15 evolutionary conserved IDPs (12 in *E. faecalis* and 3 in *E. faecium*), TimeZone is able to calculate evolutionary footprint on 10 IDP sequences. Various factors determine successful calculation of TimeZone, i.e., in-frame sequence alignment of nucleotide and protein sequences, presence of internal stop codon, presence of many sequences (>3) for multiple sequence alignment, etc. TimeZone outputs yield a number of significant features. In *E. faecais* genome, all the cell wall/ cell membrane proteins have significantly high accumulation of convergent/hotspot mutation and they exhibit signatures of positive selection in their sequences (Table 5). In *E. faecalis*, 3 out of 4 positively selected proteins belong to conserved peptidogly, can anchor motif (LPxTG) containing anchor protein present in the cell wall of gram-positive bacteria. These proteins play important roles in survival of the species through surface adhesion and are also involved during bacterial infection (Lee et al., 2001; Lévesque et al., 2005). These proteins are also believed to be involved in the development of opportunistic infection in gram-positive bacteria (Davies et al., 2009). Positive

selection of these evolutionary conserved IDR-containing proteins might be related to the pathogenic potential of these opportunistic bacteria. It is interesting to note that these LPxTG motif containing proteins were previously found to be significantly enriched in *E. faecalis* accessory genome during PPI analysis, which signifies that these groups of proteins might play crucial roles in bacterial survival through interaction with other members. In the case of *E. faecium*, 1 out of 3 conserved IDPs can be characterized by TimeZone, which shows purifying/stable selection pressure on the cytosolic protein (Table 5).

Table 5: Evolutionary selection pressure on conserved IDRs containing proteins in *E. faecalis* and *E. faecium* accessory genome

E. faecalis	**Overall Sequence Diversity**						
Gene	**# Sy n**	**# Nonsy n**	**dS (SE)**	**dN (SE)**	**dN/ dS**	**Boot - strap**	**dN/dSbased selection**
AIL03185.1	165	703	0.66 (0.11)	1.04 (0.09)	1.58	100	Positive
ARV02399.1	174	889	0.49 (0.29)	1.31 (0.22)	2.67	100	Positive
APE71703.1	165	819	0.43 (0.21)	1.27 (0.21)	2.95	100	Positive
AIL05678.1	57	117	0.59 (0.24)	1.32 (0.07)	2.23	100	Positive
ANU71609.1	107	807	0.57 (0.23)	1.31 (0.09)	2.29	100	Positive
AIL05308.1	68	102	0.20 (0.05)	0.17 (0.04)	0.85	100	Purifying
AIL05646.1	-	-	-	-	-	-	-
AIL03767.1	-	-	-	-	-	-	-
AIL04735.1	71	40	0.65 (0.23)	0.08 (0.01)	0.12	100	Purifying
AIL04689.1	44	19	0.024 (0.001)	0.002 (0.001)	0.08	100	Purifying
ANU73543.1	31	17	0.012 (0.009)	0.001 (0.003)	0.08	100	Purifying
ANU71609.1	-	-	-	-	-	-	-

E. faecium	Overall Sequence Diversity						
Gene	**# Syn**	**# Nonsyn**	**dS (SE)**	**dN (SE)**	**dN/dS**	**Boot-strap**	**dN/dSbased selection**
AQY30484.1	88	183	0.56 (0.15)	0.42 (0.09)	0.75	100	Purifying
AFK60384.1	-	-	-	-	-	-	-
AFK60351.1	-	-	-	-	-	-	-

4. Conclusion

The ten enterococcal species in the present study have diverse host distribution, ranging from the guts of humans to those of different mammals and insects. The genomes of these species vary widelyboth in genome size and GC content. Whole-genome average nucleotide identity (ANI) analysis in different enterococcal species reveal distinct characteristics in their genomes: (i) the genome of *E. faecalis* strains are found to be more homogenous to each other than *E. faecium* strains, (ii) there appears to be a conflict in taxonomic position and genomic similarity of *E.gallinarum* with E. faecium group, further analysis of which is beyond the scope of this study. Phylogenetic analysis revealed that different species of enterococci are well segregated both in their 16S and conserved core genome SNPs based phylogeny. However, in accessory genome phylogeny, it was observed that *E. faecalis* has a distinct set of accessory genes which are much different from other members of enterococcal species. Comparative structural and functional analyses of the accessory genome have been carried out in two major enterococcal species in human gut microbiota – *E. faecalis* and *E. faecium*. Functional analyses of the accessory genes reveal interesting properties of *E. faecalis* metabolic proteins. Five out of eight metabolism-specific COG categories are significantly enriched in *E. faecalis* accessory genome. However, one single class of metabolic COGs that are associated with carbohydrate metabolism is overabundant in *E. faecium* accessory genome. Characterization of the IDPs in the accessory genome of both the species revealed that *E. faecalis* has relatively large IDP sequences than *E. faecium* and these are found to interact significantly with each other through protein-protein interaction (PPI) pathways. Domain analyses of the interacting members suggest that cell wall anchor domain -containing proteins are significantly enriched in this group. During evolutionary analysis of proteins involved in PPI network, it was found that some of these proteins have conserved disordered regions in their sequences. Most interestingly, signatures of positive selection were found in some of

these cell wall anchor proteins with peptidoglycan anchor motif (LPxTG) containing groups. Evolutionary selection on an important class of signaling proteins' having stretches of conserved disordered residues is novel findings, suggesting that nature might be actively working on the adaptation of these groups of proteins, which in turn points towards the plausible evolutionary significant roles of these proteins on enterococcal adaptation to its habitat.

Acknowledgments

The authors acknowledge the computational facilities of the Bioinformatics Laboratory at Institute of Advanced Research.

Conflict of Interest

The authors declare no conflict of interest.

References

Altermann, E. (2012). Tracing lifestyle adaptation in prokaryotic genomes. Frontiers in microbiology, 3, 48.

Apweiler, R., Attwood, T. K., Bairoch, A., Bateman, A., Birney, E., Biswas, M., & Durbin, R. (2001). The InterPro database, an integrated documentation resource for protein families, domains and functional sites. Nucleic acids research, 29(1), 37-40.

Ba, A. N. N., Yeh, B. J., van Dyk, D., Davidson, A. R., Andrews, B. J., Weiss, E. L., & Moses, A.M. (2012). Proteome-wide discovery of evolutionary conserved sequences in disordered regions. Science signaling, 5(215), rs1.

Bakshi, U., Sarkar, M., Paul, S., & Dutta, C. (2016). Assessment of virulence potential of uncharacterized Enterococcus faecalis strains using pan genomic approach–Identification of pathogen–specific and habitat-specific genes. Scientific reports, 6, 38648.

Ballering, K. S., Kristich, C. J., Grindle, S. M., Oromendia, A., Beattie, D. T., & Dunny, G. M. (2009). Functional genomics of Enterococcus faecalis: multiple novel genetic determinants for biofilm formation in the core genome. Journal of bacteriology, 191(8), 2806-2814.

Bonacina, J., Suárez, N., Hormigo, R., Fadda, S., Lechner, M., & Saavedra, L. (2016). A genomic view of food-related and probiotic Enterococcus strains. Dna Research, 24(1), 11-24.

Boyd, D. A., & Mulvey, M. R. (2012). The VanE operon in Enterococcus faecalis N00-410 is found on a putative integrative and conjugative element, Tn 6202. Journal of Antimicrobial Chemotherapy, 68(2), 294-299.

Brinkwirth, S., Ayobami, O., Eckmanns, T., & Markwart, R. (2021). Hospital-acquired infections caused by enterococci: a systematic review and meta-analysis, WHO European Region, 1 January 2010 to 4 February 2020. Eurosurveillance, 26(45), 2001628.

Bullman, S., Lucid, A., Corcoran, D., Sleator, R. D., & Lucey, B. (2013). Genomic investigation into strain heterogeneity and pathogenic potential of the emerging gastrointestinal pathogen Campylobacter ureolyticus. PLoS One, 8(8), e71515.

Byappanahalli, M. N., Nevers, M. B., Korajkic, A., Staley, Z. R., & Harwood, V. J. (2012). Enterococci in the environment. Microbiology and Molecular Biology Reviews, 76(4), 685706.

Chattopadhyay, S., Paul, S., Dykhuizen, D. E., & Sokurenko, E. V. (2013). Tracking recent adaptive evolution in microbial species using TimeZone. Nature protocols, 8(4), 652.

Chaudhari, N. M., Gupta, V. K., & Dutta, C. (2016). BPGA-an ultra-fast pan-genome analysis pipeline. Scientific reports, 6, 24373.

Chen, B., Teh, B. S., Sun, C., Hu, S., Lu, X., Boland, W., & Shao, Y. (2016). Biodiversity and activity of the gut microbiota across the life history of the insect herbivore Spodoptera littoralis. Scientific reports, 6, 29505.

Davies, J. R., Svensäter, G., & Herzberg, M. C. (2009). Identification of novel LPXTGlinked surface proteins from Streptococcus gordonii. Microbiology, 155(6), 1977-1988.

de Been, M., van Schaik, W., Cheng, L., Corander, J., & Willems, R. J. (2013). Recent recombination events in the core genome are associated with adaptive evolution in Enterococcus faecium. Genome biology and evolution, 5(8), 1524-1535.

Devriese, L. A., Vancanneyt, M., Descheemaeker, P., Baele, M., Van Landuyt, H. W., Gordts, B., & Haesebrouck, F. (2002). Differentiation and identification of Enterococcus durans, E. hirae and E. villorum. Journal of applied microbiology, 92(5), 821-827.

Domann, E., Hain, T., Ghai, R., Billion, A., Kuenne, C., Zimmermann, K., & Chakraborty, T. (2007). Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic Enterococcus faecalis strain Symbioflor 1. International Journal of Medical Microbiology, 297(7-8), 533-539.

Duerkop, B. A., Palmer, K. L., & Horsburgh, M. J. (2014). Enterococcal bacteriophages and genome defense.

Dunker, A. K., Romero, P., Obradovic, Z., Garner, E. C., & Brown, C. J. (2000). Intrinsic protein disorder in complete genomes. Genome Informatics, 11, 161-171.

Emamalipour, M., Seidi, K., Zununi Vahed, S., Jahanban-Esfahlan, A., Jaymand, M., Majdi, H. & Zare, P. (2020). Horizontal gene transfer: from evolutionary flexibility to diseaseprogression. Frontiers in Cell and Developmental Biology, 8, 229.

Galloway-Peña, J. R., Liang, X., Singh, K. V., Yadav, P., Chang, C., La Rosa, S. L., & Murray, B. E. (2015). The identification and functional characterization of WxL proteins from Enterococcus faecium reveal surface proteins involved in extracellular matrix interactions. Journal of bacteriology,

197(5), 882-892.

Gao, C., Ma, C., Wang, H., Zhong, H., Zang, J., Zhong, R., & Yang, D. (2021). Intrinsic disorder in protein domains contribute to both organism complexity and clade-specific functions. Scientific reports, 11(1), 1-18.

Gilmore, M. S., Lebreton, F., & van Schaik, W. (2013). Genomic transition of enterococci from gut commensals to leading causes of multidrug-resistant hospital infection in the antibiotic era. Current opinion in microbiology, 16(1), 10-16.

Goris, J., Konstantinidis, K. T., Klappenbach, J. A., Coenye, T., Vandamme, P., & Tiedje, J. M. (2007). DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. International journal of systematic and evolutionary microbiology, 57(1), 81-91.

Guzman Prieto, A. M., van Schaik, W., Rogers, M. R., Coque, T. M., Baquero, F., Corander, J., & Willems, R. J. (2016). Global emergence and dissemination of enterococci as nosocomial pathogens: attack of the clones? Frontiers in microbiology, 7, 788.

Hammer, Ř., Harper, D. A. T., & Ryan, P. D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis–Palaeontol. Electron. 4: 9pp.

Jackson, R. W., Vinatzer, B., Arnold, D. L., Dorus, S., & Murillo, J. (2011). The influence of the accessory genome on bacterial pathogen evolution. Mobile genetic elements, 1(1), 55-65.

Jernberg, C., Löfmark, S., Edlund, C., & Jansson, J. K. (2010). Long-term impacts of antibiotic exposure on the human intestinal microbiota. Microbiology, 156(11), 3216- 3223.

Kahlke, T., Goesmann, A., Hjerde, E., Willassen, N. P., & Haugen, P. (2012). Unique core genomes of the bacterial family vibrionaceae: insights into niche adaptation and speciation. BMC genomics, 13(1), 179.

Kanehisa, M., & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research, 28(1), 27-30.

Khan, H. A., Ahmad, A., & Mehboob, R. (2015). Nosocomial infections and their control strategies. Asian Pacific Journal of Tropical Biomedicine, 5(7), 509-514.

Kim, E. B., & Marco, M. L. (2014). Nonclinical and clinical Enterococcus faecium strains, but not Enterococcus faecalis strains, have distinct structural and functional genomic features. Applied and environmental microbiology, 80(1), 154-165.

Land, M., Hauser, L., Jun, S. R., Nookaew, I., Leuze, M. R., Ahn, T. H., & Poudel, S. (2015). Insights from 20 years of bacterial genome sequencing. Functional & integrative genomics, 15(2), 141-161.

Landry, C. R., Freschi, L., Zarin, T., & Moses, A. M. (2014). Turnover of protein phosphorylation

evolving under stabilizing selection. Frontiers in genetics, 5, 245.

Lebreton, F., Willems, R. J., & Gilmore, M. S. (2014). Enterococcus diversity, origins in nature, and gut colonization.

Lee, V. T., & Schneewind, O. (2001). Protein secretion and the pathogenesis of bacterial infections. Genes & development, 15(14), 1725-1752.

Lepage, E., Brinster, S., Caron, C., Ducroix-Crepy, C., Rigottier-Gois, L., Dunny, G., & Serror, P. (2006). Comparative genomic hybridization analysis of Enterococcus faecalis: identification of genes absent from food strains. Journal of bacteriology, 188(19), 6858- 6868.

Lévesque, C. M., Voronejskaia, E., Huang, Y. C. C., Mair, R. W., Ellen, R. P., & Cvitkovitch, D. G. (2005). Involvement of sortase anchoring of cell wall proteins in biofilm formation by Streptococcus mutans. Infection and immunity, 73(6), 3773-3777.

Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., & Gordon, J. I. (2008). Evolution of mammals and their gut microbes. Science, 320(5883), 1647-1651.

Li, E., Saleem, F., Edge, T. A., & Schellhorn, H. E. (2021). Biological Indicators for Fecal Pollution Detection and Source Tracking: A Review. Processes, 9(11), 2058.

Li, W., & Wang, A. (2021). Genomic islands mediate environmental adaptation and the spread of antibiotic resistance in multiresistant Enterococci-evidence from genomic sequences. BMC microbiology, 21(1), 1-10.

Markwart, R., Willrich, N., Haller, S., Noll, I., Koppe, U., Werner, G., & Reuss, A. (2019). The rise in vancomycin-resistant Enterococcus faecium in Germany: data from the German Antimicrobial Resistance Surveillance (ARS). Antimicrobial Resistance & Infection Control, 8(1), 1-11.

Mascini, E. M., & Bonten, M. J. M. (2005). Vancomycin-resistant enterococci: consequences for therapy and infection control. Clinical Microbiology and Infection, 11(s4), 43-56.

Medini, D., Donati, C., Tettelin, H., Masignani, V., & Rappuoli, R. (2005). The microbial pan-genome. Current opinion in genetics & development, 15(6), 589-594.

Moreno, M. F., Sarantinopoulos, P., Tsakalidou, E., & De Vuyst, L. (2006). The role and application of enterococci in food and health. International journal of food microbiology, 106(1), 1-24.
Ota, H., & Fukuchi, S. (2017). Sequence conservation of protein binding segments in intrinsically disordered regions. Biochemical and biophysical research communications, 494(3-4), 602-607.

Palmer, K. L., Godfrey, P., Griggs, A., Kos, V. N., Zucker, J., Desjardins, C., & Feldgarden, M. (2012). Comparative genomics of enterococci: variation in Enterococcus faecalis, clade structure in E. faecium, and defining characteristics of E. gallinarum and E. casseliflavus. MBio, 3(1), e00318-11.

Paulsen, I. T., Banerjei, L., Myers, G. S. A., Nelson, K. E., Seshadri, R., Read, T. D., & Tettelin, H. (2003). Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. Science, 299(5615), 2071-2074.

Plotnikava, D., Sidarenka, A., & Novik, G. (2017). Antibiotic resistance in lactococci and enterococci: phenotypic and molecular-genetic aspects.

Pöntinen, A. K., Top, J., Arredondo-Alonso, S., Tonkin-Hill, G., Freitas, A. R., Novais, C. & Corander, J. (2021). Apparent nosocomial adaptation of Enterococcus faecalis predates the modern hospital era. Nature communications, 12(1), 1-13.

Ramsey, M., Hartke, A., & Huycke, M. (2014). The physiology and metabolism of enterococci.
Reid, K. C., Cockerill III, F. R., & Patel, R. (2001). Clinical and epidemiological features of Enterococcus casseliflavus/flavescens and Enterococcus gallinarum bacteremia: a report of 20 cases. Clinical infectious diseases, 32(11), 1540-1546.

Rodriguez-R, L. M., & Konstantinidis, K. T. (2014). Bypassing cultivation to identify bacterial species. Microbe, 9(3), 111-8.

Rouli, L., Merhej, V., Fournier, P. E., & Raoult, D. (2015). The bacterial pangenome as a new tool for analysing pathogenic bacteria. New microbes and new infections, 7, 72-85.

Santagati, M., Campanile, F., & Stefani, S. (2012). Genomic diversification of enterococci in hosts: the role of the mobilome. Frontiers in microbiology, 3, 95.

Semedo-Lemsaddek, T., Barreto-Crespo, M. T., & Tenreiro, R. (2009). Occurrence of putative pathogenicity islands in enterococci from distinct species and of differing origins. Applied and environmental microbiology, 75(22), 7271-7274.

Shridhar, S., & Dhanashree, B. (2019). Antibiotic susceptibility pattern and biofilm formation in clinical isolates of enterococcus spp. Interdisciplinary perspectives on infectious diseases, 2019.

Sickmeier, M., Hamilton, J. A., LeGall, T., Vacic, V., Cortese, M. S., Tantos, A., & Obradovic, Z. (2006). DisProt: the database of disordered proteins. Nucleic acids research, 35(suppl_1), D786-D793.

Solheim, M., Brekke, M. C., Snipen, L. G., Willems, R. J., Nes, I. F., & Brede, D. A. (2011). Comparative genomic analysis reveals significant enrichment of mobile genetic elements and genes encoding surface structure-proteins in hospital-associated clonal complex 2 Enterococcus faecalis. BMC microbiology, 11(1), 3.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and postanalysis of large phylogenies. Bioinformatics, 30(9), 1312-1313.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., & Kuhn, M. (2014). STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic acids research, 43(D1), D447-D452.

Tatusov, R. L., Natale, D. A., Garkavtsev, I. V., Tatusova, T. A., Shankavaram, U. T., Rao, B. S., & Koonin, E. V. (2001). The COG database: new developments in phylogenetic classification of proteins from complete genomes. Nucleic acids research, 29(1), 22-28.

Tettelin, H., Masignani, V., Cieslewicz, M. J., Donati, C., Medini, D., Ward, N. L., & DeBoy, R. T. (2005). Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial "pan-genome". Proceedings of the National Academy of Sciences of the United States of America, 102(39), 13950-13955.

Tusnády, G. E., Dobson, L., & Tompa, P. (2015). Disordered regions in transmembrane proteins. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1848(11), 2839-2848.
Uversky, V. N. (2013). A decade and a half of protein intrinsic disorder: biology still waits for physics. Protein Science, 22(6), 693-724.

Uversky, V. N., Santambrogio, C., Brocca, S., & Grandori, R. (2012). Length-dependent compaction of intrinsically disordered proteins. FEBS letters, 586(1), 70-73.

Van Der Lee, R., Buljan, M., Lang, B., Weatheritt, R. J., Daughdrill, G. W., Dunker, A. K., & Kim, P. M. (2014). Classification of intrinsically disordered regions and proteins. Chemical reviews, 114(13), 6589-6631.

Van Schaik, W., & Willems, R. J. (2010). Genome-based insights into the evolution of enterococci. Clinical Microbiology and Infection, 16(6), 527-532.

Van Schaik, W., Top, J., Riley, D. R., Boekhorst, J., Vrijenhoek, J. E., Schapendonk, C. M., & Willems, R. J. (2010). Pyrosequencing-based comparative genome analysis of the nosocomial pathogen Enterococcus faecium and identification of a large transferable pathogenicity island. BMC genomics, 11(1), 239.

Van Tyne, D., & Gilmore, M. S. (2014). Friend turned foe: evolution of enterococcal virulence and antibiotic resistance. Annual review of microbiology, 68, 337-356.

Walter, J., & Ley, R. (2011). The human gut microbiome: ecology and recent evolutionary changes. Annual review of microbiology, 65, 411-429.

Wattam, A. R., Abraham, D., Dalay, O., Disz, T. L., Driscoll, T., Gabbard, J. L., & Machi, D. (2013). PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic acids research, 42(D1), D581-D591.

Werner, G., Coque, T. M., Franz, C. M., Grohmann, E., Hegstad, K., Jensen, L., & Weaver, K. (2013). Antibiotic resistant enterococci—tales of a drug resistance gene trafficker. International

Journal of Medical Microbiology, 303(6-7), 360-379.

Willems, R. J., Top, J., Marga van Santen, D., Coque, T. M., Baquero, F., Grundmann, H., & Bonten, M. J. (2005). Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerging infectious diseases, 11(6), 821.

Wu, S., Zhu, Z., Fu, L., Niu, B., & Li, W. (2011). WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC genomics, 12(1), 444.

Wu, X., Monchy, S., Taghavi, S., Zhu, W., Ramos, J., & van der Lelie, D. (2011). Comparative genomics and functional analysis of niche-specific adaptation in Pseudomonas putida. FEMS microbiology reviews, 35(2), 299-323.

Yasmin, A., Kenny, J. G., Shankar, J., Darby, A. C., Hall, N., Edwards, C., & Horsburgh, M. J. (2010). Comparative genomics and transduction potential of Enterococcus faecalis temperatebacteriophages. Journal of bacteriology, 192(4), 1122-1130.

Zarin, T., Tsai, C. N., Ba, A. N. N., & Moses, A. M. (2017). Selection maintains signaling function of a highly diverged intrinsically disordered region. Proceedings of the National Academy of Sciences, 201614787.

Zhong, Z., Zhang, W., Song, Y., Liu, W., Xu, H., Xi, X., & Sun, Z. (2017). Comparative genomic analysis of the genus Enterococcus. Microbiological research, 196, 95-105.

Research Article

The Role of p53 mutation and copy number alterations in Osteosarcoma:An *In-silico* study

Anjani Vishnubhatla and Shuvomoy Banerjee*

Department of Biotechnology and Bioengineering, Institute of Advanced Research, Koba Institutional Area, Gandhinagar, Gujarat- 382426

Email: shuvomoy.banerjee@iar.ac.in

Abstract

Among the bone malignancies, osteosarcoma is considered the most common and has a poorprognosis. According to several studies, conventional cancer therapies for treating metastaticosteosarcoma did not show better outcomes with a low five-year survival rate (<20%). Although remarkable development has occurred in the field of cancer genetics and proteomics for the study of osteosarcoma cases, better improvement in patient survival rate was not observed due to the lack of prognostic biomarker detection strategies. Recently,tumor protein p53 (TP53) was identified as an important player as well as the "guardian of the genome" in cellular signaling and functions. Interestingly, several types of p53 mutationswere identified in human cancers. Eventually, p53 mutations were identified in the pathogenesis and progression of osteosarcoma. Apart from the p53 mutations, scientists alsoidentified a few recurrent mutations in the protein-coding genes strongly suggesting that 'somatic copy-number aberrations (SCNAs)' could be the major genetic drivers of osteosarcoma. Rational designing of an *in-silico* experimental model for cancer research involves the combination of literature surveys for biological functions with the application of computational biology to achieve the experimental hypotheses before commencing the wet lab experimental procedures. In this article, the *in-silico* analytical approachfor identifying the p53 mutation and related copy number alterations in the osteosarcoma patient cohort is shown. Such analysis will be helpful resources for conducting in-depth clinical and laboratory experiments and could be promising strategies for reducing costs for detailed wetlaboratory experimentation. In our study, different types of driver mutation frequencies and survival rates in relation with the pathogenesis of osteosarcoma by employing computational analysis are shown. Moreover, on re-analyzing the osteosarcoma patients' samples data (from Cancer Cell Line Encyclopaedia), the analysis revealed the p53 mutation types, percentage, and specific mutation sites. The *in-silico* analysis also predicted major p53 downstream signaling proteins and the network of signal transduction pathways related to osteosarcoma progression.

Keywords: osteosarcoma, copy number alteration, p53 mutation, cancer genomics

1. Introduction

Cancer burden has a profound effect on mortality and morbidity of human population worldwide. Among the bone cancers, osteosarcoma (OS) was identified as a primary malignant tumor, characterized by the clonalproliferation of sarcoma cells in the long bones (Misaghi et al., n.d.). According to a research,the overall survival rate for this malignancy was calculated as 15% along with multidrug resistance to chemotherapy (Li et al., 2015). The characterization of osteosarcoma is often associated with genomic copy number alterations and somatic p53 mutations (over 50% of human neoplasms). In this article, the types of gene mutation frequencies and survival rates related to the pathogenesis of osteosarcoma by employing in-silico experimental settings and analysis are represented. Moreover, the osteosarcoma patient samplesdata (from Cancer Cell Line Encyclopaedia from the Broad Institute and Novartis, updatedin 2019) are analyzed. Detailed analysis of the data revealed the p53 mutation types, percentage, and mutation sites on the TP53 amino acid sequences. The in-silico analysis also predicted majorp53 downstream signaling proteins and the network of signal transduction pathways related to osteosarcoma.

2. Experimental tools and methods

For database and server for the *in-silico* analysis, the cBioPortal v5.1.3 web server was used for analyzing multi-dimensional cancer genomics datasets (Cerami1 et al., 2012). This tool is a resource for the interactive exploration of several cancer attributes. The molecular profiles and clinical attributes from large-scale cancer genomics projects are available for the in- silico experimentation. The submitted patient datasets were re-analyzed to get knowledge of biological insights as well as their clinical applications.

3. Results and discussion

3.1 p53 shows high mutation frequency in osteosarcoma

This figure highlights the top contributors concerning genes associated with the prevalence of osteosarcoma in patients along with the extent to which they affect the onset of this cancer.TP53 gene is the top contributor, with a 57.90% association with the prevalence of osteosarcoma in the study sample set. Similarly, the contribution of nine other genes, namely,

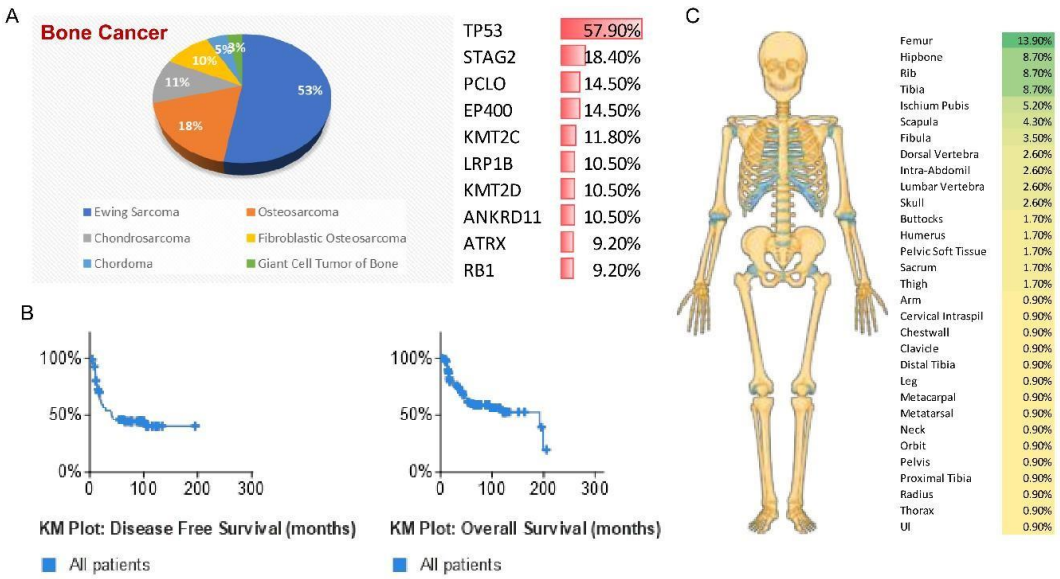


Figure 1: p53 shows high mutation frequency in osteosarcoma

(A) The types and percent of bone cancers were defined in a pie chart (B) Over survival of osteosarcoma patients were presented by the Kaplan-Meier survival analysis plots (C) The top ten genes were selected for showing the higher mutation frequencies in osteosarcoma and their relative expressions in bone tissues.

STAG2, PCLO, EP400, KMT2C, LRP1B, KMT2D, ANKRD11, ATRX, and *RB1* is mentioned in the figure, with *ATRX* and *RB1* being the least expected genes responsible for osteosarcoma progression.

Ewing sarcoma was found to be the most prevalent type of bone cancer with 53% occurrence. Most cases of Ewing's sarcoma are a consequence of reciprocal translocation between chromosomes 11 and 22, t (11, 22), triggering the EWS trans-activation domain and leading to the translation of a new EWS-FLI1 fusion protein. This fusion protein can convertsilent chromatin regions into enhancers leading to oncogenesis. It also promotes histone acetylation and the consequent chromatin relaxation makes DNA more accessible to transcription factors and enhances the expression of the associated genes (Riggi et al., 2021). Osteosarcoma was found to have 18% prevalence in the sample set. Osteosarcoma or osteogenic sarcoma arises in the primitive mesenchymal cells, causing osteoblastic differentiation and formation of malignant osteoid (Luetke et al., 2014). Abnormal extra chromosomes called small supernumerary marker chromosomes contain copies of normal chromosomes and are transcribed along with them. These are known to carry protooncogenes, contributing to the development of OS (He et al., 2018). Chondrosarcoma,with 11% prevalence is a type of bone cancer that is said to survive despite radiotherapy or chemotherapy, comprising of transformed cartilage cells. It is said to be associated with amutation in the genes that express isocitrate dehydrogenase 1 and 2 enzymes (Amary et al., 2011). Fibroblastic osteosarcoma or

fibrosarcoma, almost as prevalent as chondrosarcoma, is a cancer of fibrous connective tissue, characterized by the presence of poorly differentiatedor undifferentiated spindle cells in a storiform pattern (Augsburger et al., 2017). Chordoma (5% prevalence) and giant tumors of bone (3%) are rare types of cancers that mainly affect people in the age group of 40-70. Since chordoma is low-grade, it is mistaken to be benign; whereas the latter is an aggressive benign (non-cancerous) tumor (Figure 1. A). Disease-freesurvival refers to the period that the patient survives after primary treatment for cancer without any signs or symptoms of that cancer. Overall survival measures the time between receiving therapy and falling prey to that cancer. The survival analysis using the Kaplan- Meier estimate is the simplest way to compute the extent of survival of patients still showingsymptoms of the disease as well as completely cured patients. In the following KM plot, theX-axis represents time in months and the Y-axis represents the number of subjects that survived. The probability of survival at any given time can be computed by the specific formula (Ranstam and Cook, 2017). For each time interval, survival probability is the ratio between the no. of subjects surviving to the no. of patients at risk. The total survival probability is thus equal tothe cumulative probability until that time interval. The first plot represents data associated with disease-free survival. At the beginning of the study, there was a 100% survival of subjects. There was a drastic drop of about 50% in the number of patients surviving after the onset of bone cancer. Then with progressing time, the number of patients surviving, remainedconstant at around 40%. The second plot shows a gradual decrease in the number of patients surviving bone cancer. There was a notable drastic fall in survival percentage after 200 months of the onset of cancer in the patients (Figure 1. B). This study found out that osteosarcoma most frequently affected the femur bone at 13.90% than other bone types, namely, hipbone, rib, and tibia (which have 8.70% frequency), Ischium pubis, scapula, fibula, dorsal vertebra, intra-abdominal, lumbar-vertebra, and skull. Buttocks, humerus, pelvic soft tissue, sacrum, thigh, arm, cervical intraspinal, chest wall, clavicle, distal tibia, leg, metacarpal, metatarsal, neck, orbit, pelvis, proximal tibia, radius, thorax, and UI are the leastaffected by osteosarcoma (Figure 1. C).

3.2 In-silico analysis reveals that p53 driver mutations are associated with osteosarcoma progression

This figure provides in-depth information on the genomic make-up of the tumor using two attributes: fraction of genome altered and mutation count. Fraction of genome altered is thepercentage of the genome affected by copy number gains or losses; whereas mutation countis the number of mutations found in the tumor genome. In Pearson's correlation scatter diagrams, the correlation coefficient falls between -1 and +1. An exact value of +1 impliesa linear equation that perfectly relates X and Y which is not practical. The correlation sign depends on the regression slope (Gao et al., 2013).

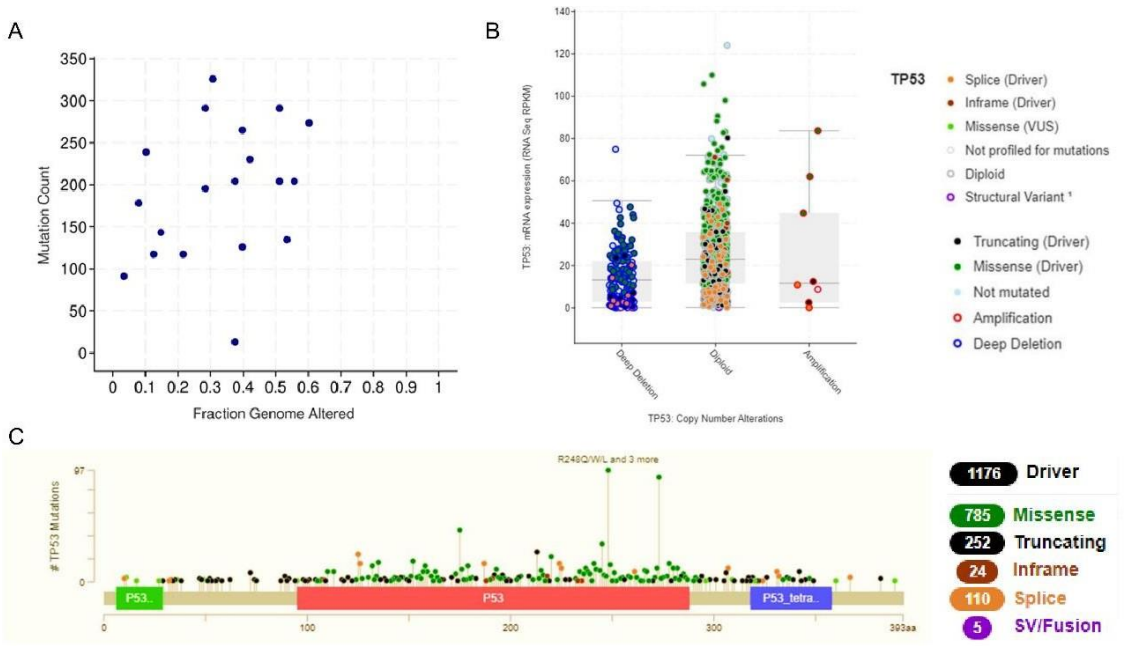


Figure 2: In-Silico analysis reveals that p53 driver mutations are associated with osteosarcoma progression

(A) The scattered plot shows the mutation frequency versus fractiongenome altered in osteosarcoma (B) Box-plot demonstrates the accumulative numbers of p53 mutations against copy number variations and transcript level (C) The number of p53 mutation sites located on TP53 protein

This Pearson correlation between the fraction of genome altered and mutation count represents a positive correlation between both attributes, with a scattered increase in alterations in the genome concerning number of mutations present. The Pearson product- moment correlation coefficient (PPMCC) or Pearson's r for the given scatter diagram may fall in the range 0<r<+1 (Figure 1. A). While the Pearson correlation describes a linear relationship between two continuous variables, the Spearman correlation also represents a monotonic relation whose coefficient is based on ranked values for each variable in place ofraw data. The given chart shows a combined Pearson & Spearman scattered diagram that represents the type of TP53 gene mutations associated with all cancer types on its horizontalX-axis and mRNA expression data for the TP53 gene as obtained from RNA seq RPKM onits vertical Y-axis. The copy number alterations under study are splice (driver), in-frame (driver), missense (VUS), diploid, structural variant, truncating (driver), missense (driver), amplification, and deep deletion. The following diagram roughly groups types of mutationsbased on their occurrence in association with other types of mutations as deep deletions, diploid, and amplifications (Figure 1. B). The figure represents the gene loci where maximum mutations are present. The coding region of the TP53 gene lying between 90 bp – 280 bp shows the maximum number of missense mutations, especially in regions near 170bp, 250 bp, and 280 bp. 110 splice mutations are shown to be scattered

along the entire gene; whereas truncating (driver) mutations are mostly found upstream and downstream of the coding region. Only 5 SV/fusion alterations have been found (Figure 1. C).

3.3 p53 transcript levels are related to copy number alterations in different age groups

This figure describes a relation between the age-associated occurrences of copy number alterations in the expressed TP53 gene. The horizontal X-axis describes the most commontype of alterations, highlighting the age of patients as a factor in their occurrence.

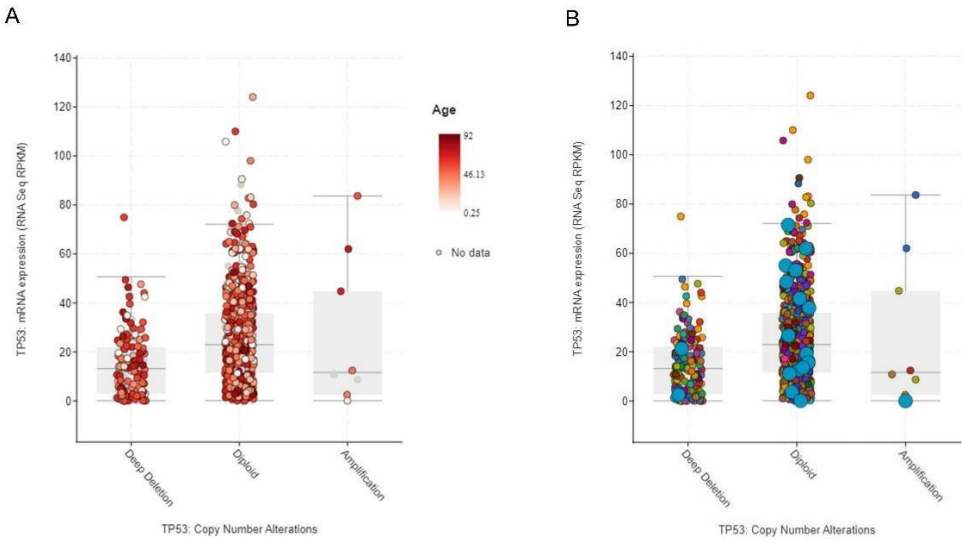


Figure 3: p53 transcript levels are related to copy number alterations in different age groups

(A) The box plot represents the data of p53 copy number variations and its transcript level about an age range (B) Same graphical representation in osteosarcoma patients (highlighted in blue circle)

The vertical Y-axis enumerates the mRNA expression data of the *TP53* gene. The maximum number of deep deletions was seen in patients approaching the age of 92. Diploid alterations were found to be most prevalent in patients above the age of 40; whereas amplifications were found mostly in elderly patients above the age of 60. Upon close inspection, diploid alterations can also be seen in infants. This data can be interpreted from the scatter diagramby analyzing the shade of red color representing the age group of osteosarcoma patients (Figure 3. A). This figure narrows down the cancer aspect in (Figure 2. B) Osteosarcoma. It describes the relationship between copy number alterations found exclusively in osteosarcoma patients and the TP53 gene expression. The blue dots highlight the prevalenceof that particular copy number alterations and its association with osteosarcoma. As shown in the figure, diploid alterations most often lead to the occurrence of osteosarcoma in a patient as compared to deep deletions and amplifications (Figure 3. B).

3.4 In-Silico prediction of p53 signaling pathways related to osteosarcoma

This figure shows major signaling pathways and to what extent they affect the main TP53 pathway leading to tumor suppression.

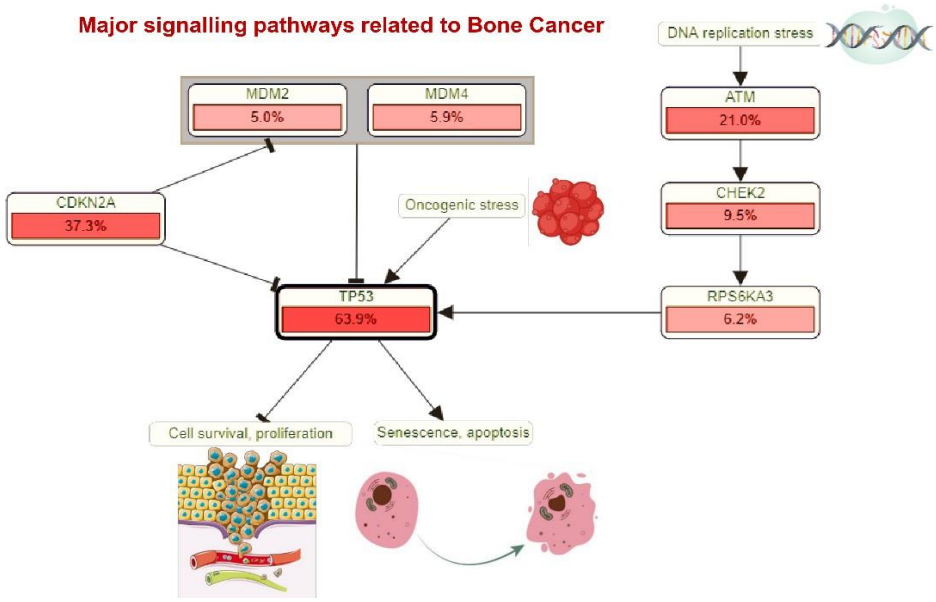


Figure 4: In-Silico prediction of p53 signaling pathways related to osteosarcoma

The illustration represents the predicted involvement of p53 signaling pathways with other molecules in osteosarcoma progression (the degree of associations was calculated in percentage by computational analysis).

Briefly, DNA replication stress as well as oncogenic stress activates TP53 transcription; whereas CDKN2A, MDM2, and MDM4 inhibit TP53 transcription. CDKN2A also inhibits MDM2 and MDM4 expression. TP53 transcription is important for a natural mode of cell death or programmed cell death, apoptosis. Inhibition of TP53 gene expression leads to tumor cell survival, consequently leading to the onset of cancer.

Mutant TP53 is considered a hopeful therapeutic target in several human malignancies including bone cancers. As the current therapeutics for cancer is facing challenges in the clinical treatment perspective, there is an unmet need to understand potential biomarkers in osteosarcomas. Computational biology mainly focuses on the use of algorithms-based models and techniques for studying cancer genomics and proteomics which could be important for providing precise in-silico predictions over laboratory wet-lab experiments and clinical trials (Yakhini and Jurisica, 2011). Therefore, the development of *In-Silico*-based techniques has come extremely useful for classifying the several genes which are strongly associated with osteosarcoma progression. In our computational studies, we aimed to analyzeand evaluate the genetic mutational significance of *TP53* in osteosarcoma patients (the datawas analyzed through cBioportal computational analysis platform). In this study,

the different mutational forms of *TP53* in osteosarcoma progression were explored. Importantly, different *TP53* driver mutations are proving direct significance in bone cancer. These studies even show the specific relation between copy number variations and transcript level of *TP53*. In particular, this *in-silico* study further emphasizes the genetic diversity among p53 mutations which may show the translational impact on osteosarcoma therapies.

4. Conclusions

In this research article, the role of different driver mutations of the p53 gene in the progression of bone cancer (Osteosarcoma) is discussed. The study is crucial for giving an in-depth comprehension of the relationship between p53 mutation and copy number alterations in osteosarcoma. The important role of p53 transcript levels on the copy number alterations in different age groups of osteosarcoma patients through in-silico analysis is shown. This study demonstrates the predicted impact of altered p53 signaling pathways and its cross-talks with other downstream signaling molecules by computational analysis. Through this *in- silico* study, various drivers of mutations and signaling pathways involving p53 in osteosarcoma are clear.

5. Acknowledgments

The author gratefully acknowledges the Institute of Advanced Research, Gandhinagar for providing infrastructure and support.

References

Augsburger, D., Nelson, P. J., Kalinski, T., Udelnow, A., Knösel, T., Hofstetter, M., Qin, J. W., Wang,Y., Gupta, A. S., Bonifatius, S., Li, M., Bruns, C. J., & Zhao, Y. (2017). Current diagnostics and treatment of fibrosarcoma –perspectives for future therapeutic targets and strategies. *Oncotarget*, *8*(61), 104638–104653. https://doi.org/10.18632/oncotarget.20136

Cerami1, E., Gao, J., Dogrusoz, U., Gross, B. E., Sumer, S. O., Aksoy, B. A., Jacobsen, A., Byrne, C. J.,Heuer, M. L., Larsson, E., Antipin, Y., Reva, B., Goldberg, A. P., Sander, C., & Schultz, N. (2012). The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer GenomicsData. *Cancer Discovery*, *2*(5), 401–404. https://doi.org/10.1158/2159-8290.CD-12-0095

Gao, J., Aksoy, B. A., Dogrusoz, U., Dresdner, G., Gross, B., Sumer, S. O., Sun, Y., Jacobsen, A., Sinha, R., Larsson, E., Cerami, E., Sander, C., & Schultz, N. (2013). Integrative Analysis of ComplexCancer Genomics and Clinical Profiles Using the cBioPortal. *Science Signaling*, *6*(269), pl1. https://doi.org/10.1126/scisignal.2004088

He, X., Pang, Z., Zhang, X., Lan, T., Chen, H., Chen, M., Yang, H., Huang, J., Chen, Y., Zhang, Z., Jing, W., Peng, R., & Zhang, H. (2018). Consistent Amplification of FRS2 and MDM2 in Low-grade Osteosarcoma: A Genetic Study of 22 Cases with Clinicopathologic Analysis. *The American Journalof Surgical Pathology*, *42*(9), 1143–1155. https://doi.org/10.1097/PAS.0000000000001125

Li, S., Sun, W., Wang, H., Zuo, D., Hua, Y., & Cai, Z. (2015). Research progress on the multidrug resistance mechanisms of osteosarcoma chemotherapy and reversal. *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *36*(3), 1329–1338. https://doi.org/10.1007/s13277-015-3181-0

Luetke, A., Meyers, P. A., Lewis, I., & Juergens, H. (2014). Osteosarcoma treatment - where do we stand? A state of the art review. *Cancer Treatment Reviews*, *40*(4), 523–532. https://doi.org/10.1016/j.ctrv.2013.11.006

M. F. Amary, M. F., Bacsi, K., Maggiani, F., Damato, S., Halai, D., Berisha, F., Pollock, R., O'Donnell,P., Grigoriadis, A., Diss, T., Eskandarpour, M., Presneau, N., Hogendoorn, P. C., Futreal, A., Tirabosco, R., & Flanagan, A. M. (2011). IDH1 and IDH2 mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours.*The Journal of Pathology*, *224*(3), 334–343. https://doi.org/10.1002/path.2913

Misaghi, A., Goldin, A., Awad, M., & Kulidjian, A. A. (n.d.). Osteosarcoma: A comprehensive review. *SICOT-J*, *4*, 12. https://doi.org/10.1051/sicotj/2017028

Ranstam, J., & Cook, J. A. (2017). Kaplan-Meier curve. *The British Journal of Surgery*, *104*(4), 442.https://doi.org/10.1002/bjs.10238

Reed, D. R., Hayashi, M., Wagner, L., Binitie, O., Steppan, D. A., Brohl, A. S., Shinohara, E. T., Bridge, J. A., Loeb, D. M., Borinstein, S. C., & Isakoff, M. S. (2017). Treatment pathway of bone sarcoma in children, adolescents, and young adults. *Cancer*, *123*(12), 2206–2218. https://doi.org/10.1002/cncr.30589

Riggi, N., Suvà, M. L., & Stamenkovic, I. (2021). Ewing's Sarcoma. *The New England Journal ofMedicine*, *384*(2), 154–164. https://doi.org/10.1056/NEJMra2028910
Yakhini, Z., & Jurisica, I. (2011). Cancer computational biology. *BMC Bioinformatics*, *12*, 120.https://doi.org/10.1186/1471-2105-12-120

Review Article

Security and privacy concerns, Medjacking, and attacks in IoT Healthcare System

Mukesh Choubisa*[1], Raghav Joshi[2]

[1,2] *Institute of Advanced Research (IAR),*

Koba, Institutional Area, Gandhinagar 382426, India

**Corresponding Author: mukesh24sa@gmail.com*

Abstract

The Internet of Technology (IoT) is an emerging technology in computer science society. IoT, internet based information architecture facilitate the exchange of information from one place/system to another place/system. The internet of technology has the principle of providing an IT-infrastructure to exchanges of 'things' in a reliable and secure manner in network. The Internet of Things (IoT) refers to a basic concept of linked/connected devices of all types over the internet, wireless or wired. The popularity of IoT has improved rapidly as these technologies are used by many organizations for various purposes, including medical devices, network communication, education, business development, and transportation.

Keywords: - IoT security, IoT privacy, IoT in healthcare, medjacking in IoT, IoT healthcare security and privacy challenges, IoT healthcare systems, security of IoT, privacy, information security.

1. Introduction

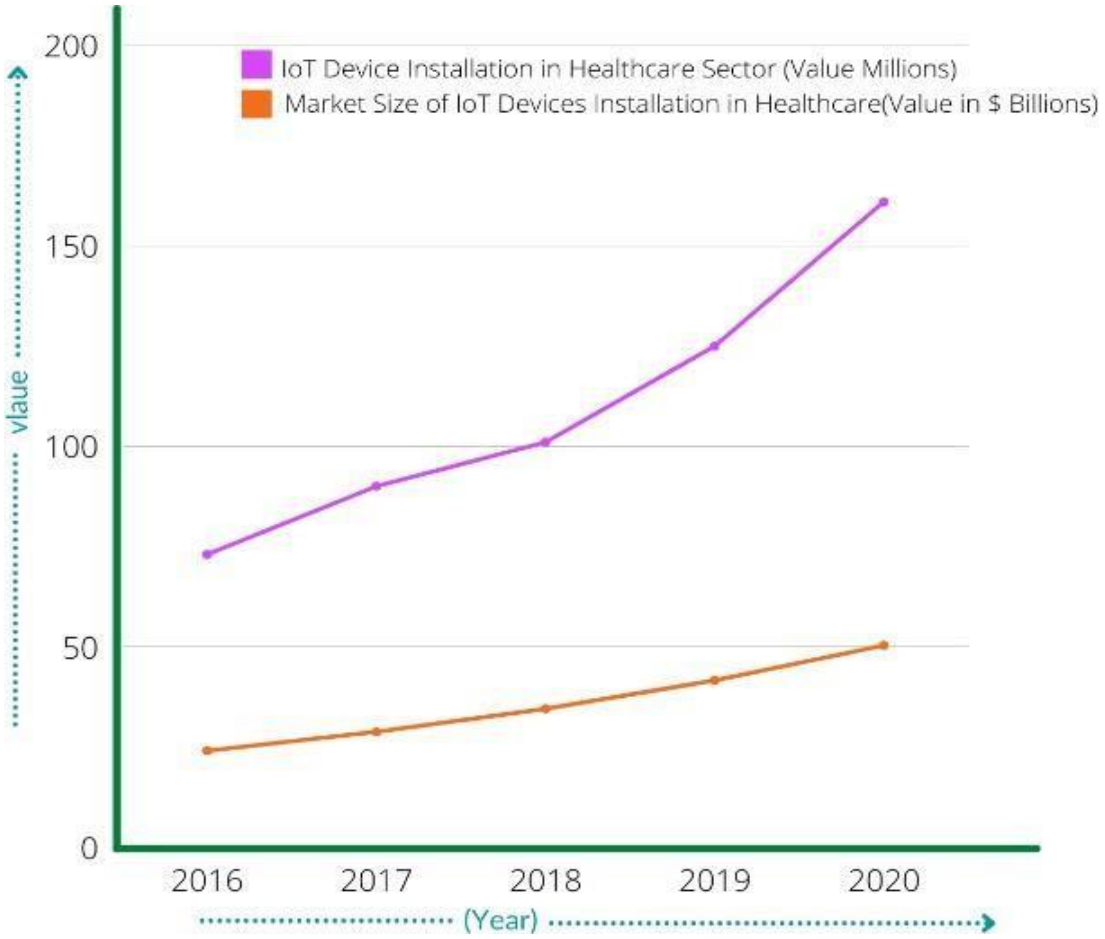
IoT term was invented by Kevin Ashton in the year 1999. The main goal of IoT was for promoting the concept of Radio Frequency Identification (RFID) RFID includes sensors, actuators, and program counter. However, the original idea of IoT was discovered in the 1960s. In 1960, IoT was known as pervasive computing or embedded Internet.

The Internet of Things (IoT) envisions connecting everything to everyone and everything using identification technologies. The identification technology includes voice recognition, smart cards, biometrics, sensors, and bar codes which are connected by wired or wireless systems. This vision will offer a unique perspective on the opportunities and challenges associated with IoT.

The main concept behind IoT is to highlight the interconnection between reality/ realism and physical world via internet. IoT adheres to the hyper connectivity notion, which denotes that businesses and individuals can connect or communicate with one another from anywhere in the world. IoT deals in industry with wide range of software/application such as transportation, smart cities, agriculture,

emergency services, medical services, education sector services, and logistics.

The IoT is the collection of devices that are both connected as well as smart. The IoT devices arerooted with hardware, software, sensors, and network connectivity. IoT network connectivity enables collection and exchange of data via network. IoT application can be controlled remotely across worldwide network infrastructure locations; this creates an opportunity for more direct integration of devices into IoT network which results in more improvement, efficiency, and accuracy.



Graph 1: The growth and market value of IoT devices in healthcare sector

With several applications, including integrated mobile medication devices and remote monitoring, the healthcare sector is one of the IoT's fastest-growing industries. Patients can monitor their blood pressure, diet, pulse, and fitness with the use of health-monitoring equipments, and they can also get real-time feedback from hospitals and doctors. These identification devices are integrated with smart healthcare systems and function in unison with a network to gather and transmit patient data digitally.

IoT healthcare system requires a healthcare bionetwork which include human, process, and

technology. In general, there are four main components of IoT Healthcare System which is data, devices, human and process. Data represents all the health information obtained and stored in an IoT Cloud. Devices consist of all medical equipments that are built IoT-ready; while people refer to all stakeholders in the healthcare practice such as doctors, patients, and all types of medical practitioners. Finally, the health-related process represents namely, Care Delivery, Wellness, and Preventive Care. Figure 1 represents how an IoT platform works in healthcare system.

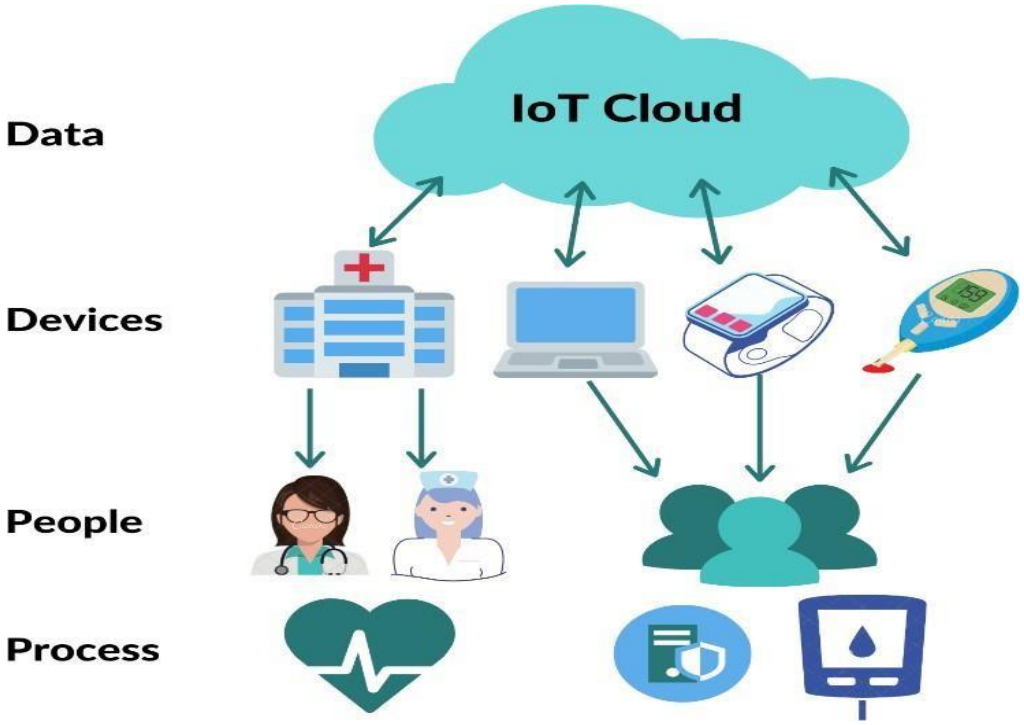


Figure 1: IoT platform for Healthcare System

1. Medical Internet of Things (MIoT)

The medical Internet of Things is a collection of connected devices used in the healthcare industry to carry out tasks and offer services. A new technology called MIoT is emerging in computer engineering for e-healthcare. By using wearable technology or sensors in body, MIoT devices gather important patient body characteristics and keep track of their medical information. Better health has been made possible by MIoT, which supports a wide range of applications from wearable technology to body-implanted sensors. Perception layer, network layer, and application layer are the three levels that make up the typical MIoT structure, as depicted in Figure 2.

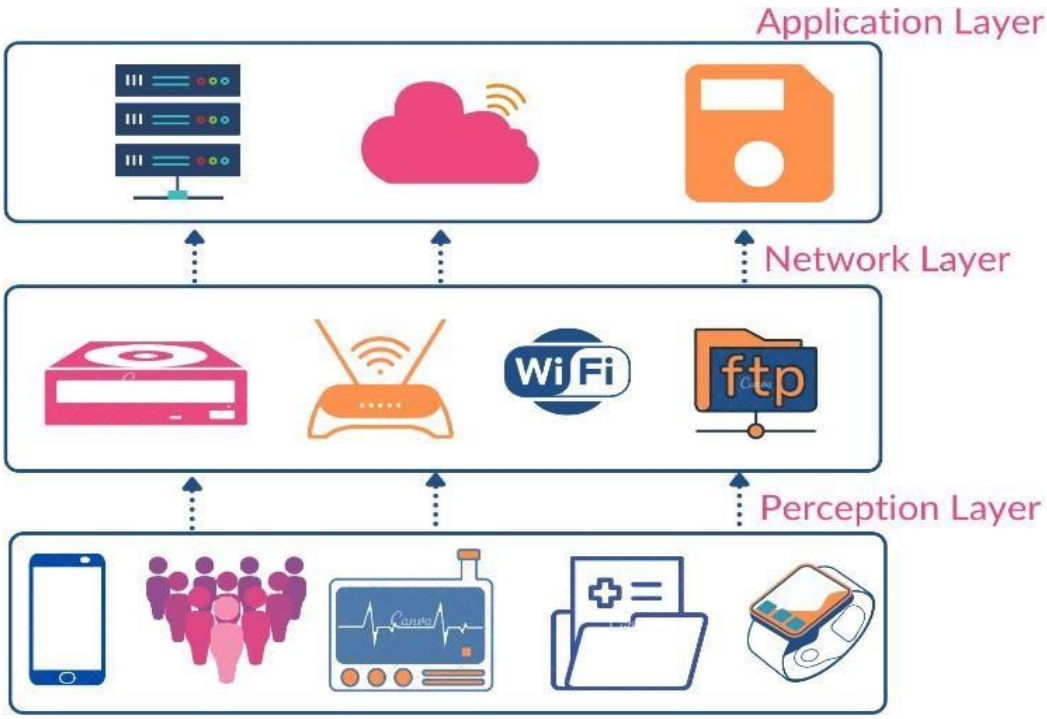


Figure 2: Structure of medical internet of things

The main role of the perception layer is to collect data from human with the help of IoT enabledmedical devices. The network layer, which is composed of wireless and wire system, processes and sends/submits the i/p data received by the perception layer. Network layer works with OSI transport protocols layer for transmission of data and reducuction of energy consumption, and ensures security and privacy of human data. The application layer integrates the information resources to provide medical services and fulfill users' needs.

2. Security and privacy concerns

IoT is the networking of wired devices that are connected to each other to serve a specific purpose. With the help of various software and sensors, these devices manage to share their data. The necessity of IoT has been increasing exponentially as its application covers various sectors and tenacities including education, infrastructure, business development, daily communication, and transportation. As these IoT devices consists of sensors and actuators, the technology becomes a part of cyber-physical systems where the control of mechanism lies in the algorithm used to develop the embedded systems. There is a huge variety of available IoT applications, ranging from home appliances such as lighting, colling/heating systems, and microwave ovens to large organizations including governments for smart parking, tolls, e-challans, etc. According to experts, at least 30 billion devices will be connected to IoT networks by the year 2025.

Fortunately, a substantial portion of these will be having medical applications consisting of daily

gadgets such as fitness band and health trackers, to medical equipment such as pacemakers and cochlear implants, to hospital scale devices such as infusion pumps, ventilators and vital tracking monitors, mobile medical workstations and ambulances.

The cyber security risks and unwanted access into IoT devices have seen a growth leading to misuse of data and losing of information eventually, resulting in crisis. Such inappropriate security attacks increase the chances of data breaches and other threats which may encounter in loss of data, property, or human life. The primary reason of such an attack is weak security policies which happen to be easily intercepted during wireless transfer.

In late 2015, a team of two security researchers exposed over 68,000 medical systems that were available unprotected online and 12,000 of them belonged to a single healthcare organization. The most troublesome of it was that most of those integrated devices found had their computers running expired versions of Win-XP known to have lots of exploitable vulnerabilities. All of these devices were easily discoverable via Shodan, a search engineusually used to find internet connected devices online and are also easy to hack via brute-force attacks and using hard-coded logins.

During their research, these two infosec experts found aesthesia equipments, cardiology devices, nuclear medical systems, infusion systems, pacemakers, MRI scanners, and communications gear, all via simple Shodan queries. This is not the sole instance which may help determine the feebleness with privacy and security of IoT oriented devices. Any erroneous mindset might have led to deception of these medical equipments resulting in loss of human life, thus acting as a life taking weapon.

2.1 Security issues

It is clear now that IoT is quite diverse from traditional computers and computing devices making it more vulnerable to security challenges in various ways:

- Many devices in the IoT are produced for deployment on a huge scale. The perfect example of this is sensors.
- Usually, the deployment of IoT embraces a set of alike or nearly identical appliances that bear similar characteristics. This similarity amplifies the magnitude of any vulnerability in the security that may significantly distress many of them.

This obviously helps to decode the rise in data security and liability risks in the healthcare sector because of IoT. Another major factor adding to this risk is that these IoT devices act on their own (automatically) without human intervention making it more vulnerable to control by end-users.

One such example is of implantable cardioverter-defibrillator (or ICD) programmed monitors used by doctors to monitor a patient's heart condition conveniently from anywhere inside the hospital or within the stipulated range defined for the device. These IoT embedded ICD devices can deliver data and statistics about a person's heart rhythms to a doctor. In case of abnormal rhythm, as required, the doctor may portably send signals to generate a specific amount of electrical shock to get the heart

beating properly. Researchers have been able to demonstrate how a malicious hacker can trigger the device to malfunction or to intercept the signals from both the ends delivering a dangerous shock, all of this without being caught.

2.2 Privacy

Healthcare data is collected from IoT devices on a regular basis. This process of information gathering takes place through remote access mechanisms which have some challenging effects on the privacy and security. Here, the data being collected by embedded sensors are transmitted to the required destination via the same internet through which the IoT devices are connected and communicated with each other. Thus, here the vulnerability lies in losing of the health data over the process of transmission. Additionally, similar sorts of healthcare data are collected from different health units which in turn share them with other departments. Thus, it should be the utmost priority to secure these data and ensure that your network is leak proof, because healthcare data includes essential significant information about one's health condition. The primary perspective of the utility of the IoT is solely dependent on how well it can respect the privacy choices of people. It is a prerequisite to know that the rights of privacy and user's privacy should always be protected under the service offered by the IoT device manufacturing company.

Since data is transmitted online preferably through wireless networks, protecting the confidentiality and integrity of patients' records are important to ensure the correct treatments are given to the correct patients.

Recent research suggested that the primary objective of any attacker is to take hold of one's health issues in order to blackmail or threaten that person. Therefore, the privacy of data must be protected at any cost to avoid any mis-happenings.

As perfectly mentioned by Tarouco, there are five main risks of IoT implementation inhealthcare, namely (1) Risk of patients' privacy exposure (2) Threats of cyber-attacks on privacy (3) Data eavesdropping and data confidentiality (4) Identity threats and privacy of stored data (5) Location privacy. However, distracted by the new features and capabilities of IoT, requirements for security and data privacy aspects have been gradually unheeded.

Figure 3: Top 5 risks of IoT implementation in healthcare sector

2.2 Privacy issues

The most common issues related to privacy of one's data are mentioned below as:

1. **Risks of patients' privacy exposure**

The primary privacy issue is to keep the patient's health data safe and confidential. A Personal Health Record Data (PHRD) is an individual electronic record of health-related information that conforms to the nationally recognized interoperability standards. PHRD is pinched from multiple sources and is directly reported to electronic healthcare centre. Because they include personal information, they can become the target of cyber-attacks that result in the theft of private data.

2. **Data eavesdropping**

Basically, patient's health data are available only to authorized persons and organization. However, such data can be out while flowing over the wireless links. For example, IoT-based insulin delivery system uses wireless communication links which are frequently used to launch privacy attacks and therefore needs sufficient protection of the transferred data.Ownership of data. Every country has laws to protect its people's health data, but government laws vary from state to state. Moreover, in certain cases, such as in case of fitness wearable devices, many people would think that the data tracked and collected is bound to be protected by government law but in many cases it is not.

3. **Location privacy**

Location privacy is all about eavesdropping on a patient's whereabouts. Location privacy in WSNs, specifically hiding the message sender's location, can be achieved through routing to a randomly selected intermediate node (RRIN).

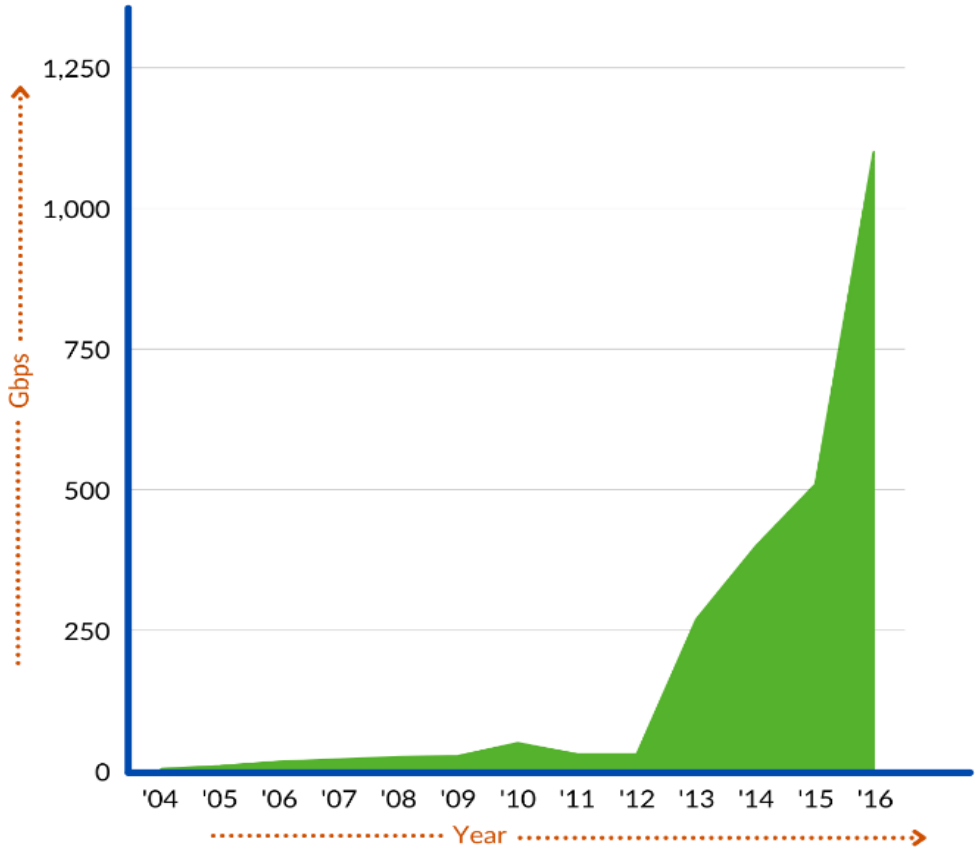
3. Attacks

3.1 Distributed Denial of Service (DDoS)

Distributed denial of service (DDoS) is an attack where multiple compromised systems are used to target a single system causing a denial of service and eventually resulting that system to crash. To understand better, a hacker temporarily manages to take control of a number of internet-enabled devices into an arrangement regarded as botnet and then make simultaneous requests to a server or a group of servers for a specific service, thereby overwhelming the server with bogus requests and make it ignore legitimate requests from end-users.

A hacker can do this for different reasons. Earlier it was for bragging rights and financial gains, but in today's world, it may be used to take control of any hospital server and extract the database or disrupt the working efficiency. For an instance, Anonymous, a decentralized group of hackers besieged the Boston's Children's Hospital with a DDoS attack after the hospital firmly asked one of their patients, a 14-year-old girl, to be admitted as a ward of the state and that custody be taken away from her parents. The doctors of BCH believed the child's ailment was actually a psychological illness and that her parents were pushing for unnecessary treatments for a disorder the child did not have. This was followed by a custody debate surrounding Boston Children's Hospital in the middle of this controversial case and some, including members of Anonymous, viewed this as an infringement on the girl's rights, therefore steering DDoS attacks against the hospital's network which resulted in attack on others on that network too, including Harvard University and all its hospitals to lose internet access as well. The networks experienced outages for almost a week and some medical patients and medical personnel could not use their online accounts to check appointments, test results, and other case information, according to the Boston Globe. As a result, the hospital spent more than $300,000 responding to and mitigating the damage from this attack, according to the attacker's arrest affidavit.

Remark 1: The below graph represents the statistics of the largest attacks faced by the hospitals of United States of America in terms of incoming traffic rate represented through Gigabits per second on a yearly basis between the years 2004 and 2016. (1Gbps = 10^6Kbps or 10^9 bps)



Graph 2: The biggest DDoS attacks on hospitals encountered each year

Some of the individual connected medical devices like pacemakers can only send statistics information while others hold the power to send as well as receive data. This may leave the patients vulnerable to a hacker trying to harm (or kill) them or use their device as a portal to access medical data without any risk of getting caught.

Billy Rios, a security researcher who helps the US Department of Homeland Security proved he could remotely administer a lethal dose of drugs through a patient's insulin pump. Following this, his team was ultimately able to figure out the passwords. Since then, the Food and Drug Administration (FDA) which is assigned to regulate the sale of medical devices, has been helping out with formal guidelines on the issue. They have also been publishing new recommendations on how medical device makers should take cyber-security attacks into account.

3.2 Medjacking

Figure 4: Pictorial representation of Medjacking

Medjacking, as the name suggests is the process of hijacking the biomedical devices available in hospitals in order to create backdoors with the intention to harm and/or threaten a patient. In history, this process of attack has been commonly referred to as 'a ticking time bomb' and the threat is considered so dangerous that the FDA, HITECH and HIPPA (US) have been constantly working to counter measure and eradicate such disasters.

Healthcare institutions continue to remain attractive targets because of all of the internet-connected systems and medical devices. This presents an attacker with a highly connectedcommunity that brings these vulnerable medical devices together with high value patient data. All it takes is one successful attempt for the attacker to establish a backdoor, find and steal data, or use automated tools to set a ransomware attack in motion.

The list of devices vulnerable to MEDJACK and MEDJACK.2 is very large. This includesdiagnostic equipment (PET scanners, CT scanners (infusion pumps, medical lasers, and surgical machines), life support equipment (heart - lung machines, medical ventilators, extracorporeal membrane oxygenation machines, and dialysis machines) and many more. As noted above, the majority of these devices use conventional, often older operating systems and proprietary internal software developed specifically for medical devices.

TrapX recently released a report providing details about incidents of medjacking (MEDJACK.2) stating its reference in three hospitals. For an instance, with the case of one hospital, the attackers infested multiple medical machines including Radiation Oncology System, Trilogy LINAC Gating System (both of them used in treatment of cancer) and the Fluoroscopy Radiology System (Fig. 5). Reports by TrapX suggest that they used two various forms of malware on these old and vulnerable Windows XP systems to steal passwords to access other systems in the hospital, along with some confidential data belonging to the hospital association.

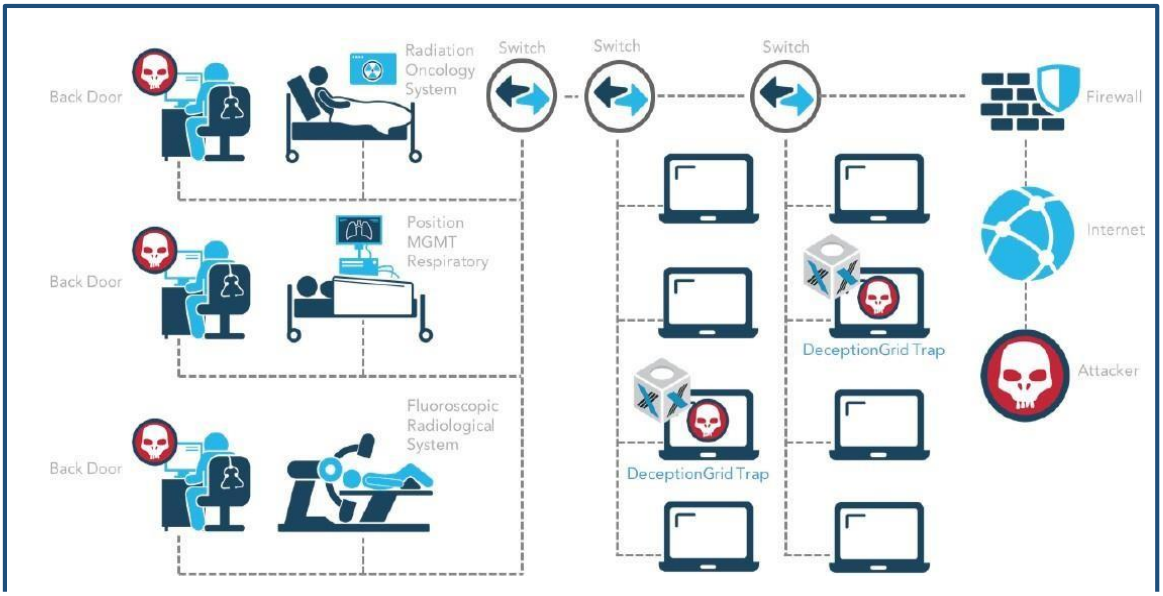


Figure 5: Diagrammatical representation of attack process (Source: TrapX)

Insulin pumps are medical devices that automatically deliver required dose of insulin to the body at a frequency predefined by the doctor. It also holds the functionality to release insulin in the body in case of irregular change in body sugar levels to prevent any sorts of medical emergencies relating to it.

The Animas One Touch Ping and Medtronic are two of the companies that sold these Insulin Pumps with a feature of wireless remote control that patients could use to order the pump to deliver a dose of insulin without awkwardly pressing buttons on the attached device under the clothes. The hackers, using signal-boosting equipments and brute- force technique, managed to get into the insulin pump system and thus control the pump through their application (Fig. 6). All they had to do is generate a specific frequency to overdose the patient with insulin before the machine could detect security issues and alert the patient.

Figure 6: Screenshot of attackers' computer (Source: QED Security Solution [10])

Medjacking has been continuously evolving adding newer layers of camouflage to the strategies of the attackers. Through such attacks, the attackers hold the power to launch cyber-attacks on hospitals, and exfiltrate data leading to breach in data integrity and resulting in probable manipulation in an individual's health.

Clearly, biomedical devices have a profoundly beneficial impact on the quality of healthcare. But medjacking is an inescapable and grave threat and traditional security solutions being offered are not proving to be effective enough for IoMT security, and thus requires a proper attention to overcome all the security challenges.

4. Preventive approach to overcome attacks

All sorts of security measures need to be incorporated while designing the IoT device keeping in mind all forms of risk and strategy to overcome them. Some of the primary facts to be ensured are implementation of Privacy Enhancing Technologies (PET), improvisations to new data encryption techniques as well as to establish some strict access control to avoid jeopardy of such active and passive attacks from happening.

Privacy enhancing techniques include ways to fulfill the customer's privacy requirements by various implementations in technology such as Virtual Private Networks (VPN), Transport Layer Security (TLS), DNS Security Extensions (DNSSEC), and Private Information Retrieval (PIR) System.

Cryptography is a technique where a plain text is transformed into cipher text using numerous encryption algorithms. The message transmission takes place through public channel making it important to generate a cipher text. Data encryption can be done via three levels of communication

mode: (1) link encryption, (2) node encryption, (3) end-to-end encryption.

In link encryption, the message received from the former link is decrypted into plain text and plain text to cipher text by with the help of secret key of next link. Node encryption does not allow messages in plain text form in the network node. Thus, node encryption can provide more security for network. When using end-to-end encryption, the message is not decrypted until the destination node has received it. Because messages arew'??:q3reduce always present as cipher-text throughout the transmission process and there is no leakage of information, even any node is corrupted.

Access control refers to the mechanism through which a data system is cre ated containing identity of a user along with some set of rules and policies to prevent access to its resources by any unauthorized user. Some common encryption methods applied in access control include symmetric key encryption, asymmetric key encryption, and attribute-based encryption.

5. Conclusion

A variety of medical devices along with software related to healthcare sectors are being developed and utilized to expand the eminence of medical services in monitoring and curing the patients. Thus lately, the demand and innovation of various IoT devices and its application in this sector has seen an exponential growth. These devices primarily deal with private information of individual's healthcare, including their vitals and past history of their medical conditions. This may turn out to be a disadvantage giving the attackers a chance to control one's body's health by modifying medical equipment and devices. Therefore, it is important to identify the features of security requirements in IoT healthcare segments, followed by some precise and sensitive steps to overcome all the vulnerabilities and make IoT a safer environment for medical sector.

References

A. Chacko and T. Hayajneh, "Security and Privacy Issues with IoT in Healthcare,"*EAI Endorsed Trans. Pervasive Heal. Technol.*, vol. 4, no. 14, pp. 1–7, 2018, doi: 10.4108/eai.13-7-2018.155079.

F. I. Salih, N. Azaliah, A. Bakar, N. H. Hassan, F. Yahya, and N. Kama, "IOT Security Risk ManagementModel for Healthcare Industry (Special Issue 2019) pp.131-144 IOT SECURITY RISK MANAGEMENT MODEL FOR HEALTHCAREINDUSTRY," pp. 131–144, 2019.

W. Sun, Z. Cai, Y. Li, F. Liu, S. Fang, and G. Wang, "Security and Privacy in the Medical Internet of Things: A Review," *Secur. Commun. Networks*, vol. 2018,2018, doi: 10.1155/2018/5978636.

"Thousands of IoT Medical Devices Found Vulnerable to Online Attacks."https://news.softpedia.com/news/thousands-of-iot-medical-devices-found-vulnerable-to-online-attacks- 493144.shtml (accessed Mar. 20, 2021).

D. Thilakanathan, Y. Zhao, S. Chen, S. Nepal, R. A. Calvo, and A. Pardo,"Protecting and Analyzing Health Care Data on Cloud," in *Second International Conference on Advanced Cloud and Big Data*, 2014, pp. 143–149, doi: 10.1109/CBD.2014.25.

C. Eken and H. Eken, "Security Threats and Recommendation in IoT Healthcare,"*Proc. 9th EUROSIM Congr. Model. Simulation, EUROSIM 2016, 57th SIMS Conf.Simul. Model. SIMS 2016*, vol. 142, pp. 369–374, 2018, doi: 10.3384/ecp17142369.

Tarouco *et al.*, "Internet of Things in healthcare: interoperability and securityissues," in *conference on communications (ICC)*, 2012, pp. 6121–6125.

"DDoS Attacks: In the Healthcare Sector." https://www.cisecurity.org/blog/ddos-attacks-in-the-healthcare-sector/ (accessed Mar. 20, 2021).

T. R. Labs, "MEDJACK.2 Hospitals under Siege," pp. 1–30, 2016.

"Hackers Made an App That Kills to Prove a Point | WIRED." https://www.wired.com/story/medtronic- insulin-pump-hack-app/ (accessed Mar. 20, 2021).

R. H. Weber, "Internet of Things - New security and privacy challenges," *Comput.Law Secur. Rev.*, vol.26, no. 1, pp. 23–30, 2010, doi: 10.1016/j.clsr.2009.11.008.

L. Tawalbeh, F. Muheidat, M. Tawalbeh, and M. Quwaider, "IoT privacy and security: Challenges and solutions," *Appl. Sci.*, vol. 10, no. 12, pp. 1–17, 2020, doi:10.3390/APP10124102.

S. Nasiri, F. Sadoughi, M. H. Tadayon, and A. Dehnad, "Security requirements of internet of things-based healthcare system: A survey study," *Acta Inform. Medica*, vol. 27, no. 4, pp. 253–258, 2019, doi: 10.5455/aim.2019.27.253-258.

X. C. Yin, Z. G. Liu, B. Ndibanje, L. Nkenyereye, and S. M. R. Islam, "An iot-basedanonymous function for security and privacy in healthcare sensor networks," *Sensors (Switzerland)*, vol. 19, no. 14, Jul. 2019, doi: 10.3390/s19143146.

K. Angrishi, "Turning Internet of Things (IoT) into Internet of Vulnerabilities (IoV): IoT Botnets," *arXiv*, pp. 1–17, 2017.

M. Gloukhovtsev, "Iot Security: Challenges, Solutions & Future Prospects," pp.1–44, 2018,[Online]. Available:https://education.dellemc.com/content/dam/dell- emc/documents/en-us/2018KS_Gloukhovtsev- IoT_Security_Challenges_Solutions_and_Future_Prospects.pdf.

D. Dziak, B. Jachimczyk, and W. J. Kulesza, "IoT-based information system forhealthcare application: Design methodology approach," *Appl. Sci.*, vol. 7, no. 6, 2017, doi: 10.3390/app7060596.

W. Boonyanusith and P. Jittamai, "Transforming Blood Supply Chain Managementwith Internet of Things Paradigm Transforming Blood Supply Chain Managementwith Internet of Things Paradigm," *Proc. Hambg. Int. Conf. Logist.*, vol. 23, p. 23,2017, [Online]. Available:https://pdfs.semanticscholar.org/855d/163941e536b165ada2fc739160a5a68cd5a8.pdf.

"Hackers create more IoT botnets with Mirai source code | PCWorld." https://www.pcworld.com /article/3132571/hackers-create-more-iot-botnets-with- mirai-source-code.html (accessed Mar. 20, 2021).

"Healthcare's 'Internet of Things' should be the 'Security of Things' | Healthcare IT News." https://www. healthcareitnews.com/blog/healthcares-internet-things- should-be-security-things (accessed Mar. 20, 2021).

"Medjacking: The newest healthcare risk? | Healthcare IT News." https://www.healthcareitnews.com/news/medjacking-newest-healthcare-risk (accessed Mar. 20, 2021).

"Medjacking–An Epidemic in Healthcare | Healthcare IT News." https://www.healthcareitnews.com/sponsored-content/medjacking-–-epidemic- healthcare (accessed Mar. 20, 2021).

"IoT in Healthcare: Use Cases, Trends, Advantages and Disadvantages | ExistekBlog." https://existek.com/blog/iot-in-healthcare/ (accessed Mar. 20, 2021).

"MEDJACK: Hackers hijacking medical devices to create backdoors in hospital networks Computer world." https://www.computerworld.com/article/2932371/medjack-hackers-hijacking-medical-devices-to-create-backdoors-in-hospital-networks.html (accessed Mar. 20, 2021).

"Medjacking How Hackers use Medical Devices to Launch Cyber Attacks." https://www.meddeviceonline.com/doc/medjacking-how-hackers-use-medical-devices-to-launch-cyber- attacks-0001 (accessed Mar. 20, 2021).

"Ensuring privacy and security in the healthcare IoT | by Sciforce | Sciforce Medium." https://medium.com/sciforce/ensuring-privacy-and-security-in-the- healthcare-iot-7b97549d629c (accessed Mar. 20, 2021).

"J&J warns diabetic patients: Insulin pump vulnerable to hacking | Reuters." https://www.reuters.com/article/us-johnsonjohnson-cyber-insulin-pumps-e- idUSKCN12411L (accessed Mar. 20, 2021).

Review Article

Insomnia and sleep disorders: Recent therapeutic updates

Juni Banerjee*

Department of Biotechnology and Bioengineering,

Institute of Advanced Research (IAR),

Koba Institutional Area, Gandhinagar, Gujarat- 382426

Email: juni.banerjee@iar.ac.in

Abstract

'Sleep' is a key physiological process associated with the fine changes in the brain's neuro-physiological activities and also with specific patterns of a human being's sub-consciousnessand memory. The human brain creates fast 'alpha' waves related to mental relaxation and then a slow wave of sleep called 'theta' waves crucial for learning and memory. However, cases of insomnia and other sleep disorders are escalating in the current times even though several cases remain under the criteria of not diagnosed and untreated. Physicians often prescribe nonbenzodiazepine hypnotics (i.e., z-drugs) and anti-depressants as the first-line medications for insomnia. However, the cons of using the z-drugs are parasomnias and central nervous system depression. Second-line drug therapy includes melatonin and suvorexant out of which melatonin is one of the safest available medications. Medications like benzodiazepines, antihistamines, and antipsychotics are not so effective and even safe. Hence, increased use of nonpharmacologic treatment as an alternate and safe approach is more being considered. This includes cognitive-behavioral therapy intervention, integrative medicine, mindfulness, and meditation.

Keywords: sleep disorder, sleep medicine, insomnia, stress, depression

1. Introduction

Regular sleeping habit makes us physiologically fit and active throughout the day. Sound sleep depends upon two different and overlapping neuro-physiological phenomena (Cross et al., 2018). In natural conditions, the body's homeostatic mechanism tries to put increasing pressure on getting asleep after one awake cycle and the body's circadian rhythm always keeps us alert during the daytime and makes us sleepy at night when the light intensity is low (Potter et al., 2016). In our modern and fast life, sometimes we must work long hours and often we face stress and anxiety and lack of physical exercise which may contribute to manifesting the symptoms of sleep disorders. In addition, manipulation of the light levels and intensity disrupts natural circadian rhythm which also aids in the process of sleep apnea(Dijk and Landolt, 2019). Especially the advent of the industrial

revolution and in recent times, the digital revolution is affecting our normal sleep cycle. Therefore, the cases of insomnia and other sleep disorders are increasing rapidly although most of the cases remain under- diagnosed and under-treated. Moreover, there are many attributes for increasing insomnia in those people who have mental or physical health-related problems. In this review, several aspects of insomnia and current therapeutic strategies are discussed.

2. Functions of sleep

The exact function, advantages, and quintessentially of sleep are still yet to be known. Eventhough there are several theories, the most common belief about sleep is that it primarily helped our ancient ancestors to survive, e.g. by not moving for a long time ancestors pretended themselves to be dead and avoided getting caught by predators. This may seem partially true at night times when the vision is generally weaker. However, sleep practically makes a person less alert and aware of their surroundings, hence making it harder to escapefrom predators. The notion that sleeping might help to store energy seems the next possibletheory [13]. This theory also does not 100% fit, as because even if our bodies use less energywhile sleeping, the difference between being awake and asleep isn't extremely different. Probably the well-supported theory about sleep being a requisite to all of us is that sleep helps our body to effectively grow, heal, and repair itself [14]. Our body is better at attacking intruders and fixing itself when given rest, rather than expending all the energy walking, talking, and working. Moreover, it has been revealed that sleep plays a key role in building as well as strengthening one's memory [15].

3. Mechanism of sleep

During sleep, we generally remain very less responsive to external stimuli including light, temperature, sound, etc. According to a research, sleep is considered a reversible process (Holst and Landolt, 2015). Sleep behavior is controlled by homeostatic regulators with specificcompensation set-points. Interestingly, sleep is a highly organized and specialized behavior observed in higher vertebrates (Cirelli and Tononi, 2008). In this case, lower vertebrates, likefish shows much undefined sleeping patterns (Kelly et al., 2019). More interesting patterns were identified in vertebrates; including worms, crayfish, and fruit flies who do not show proper 'sleep' behavior instead they remain active in the '*high arousal state*' and exhibit inactivity in the '*low arousal state*' (Bushey et al., 2011). This is interesting to know that therange of sleeping hours varies in the animal world. It was estimated that a giraffe requires only 1.9 hours of sleep in a day, whereas a brown bat requires 19.9 hours of sound sleep (Rattenborg et al., 2019). To find out the precise molecular mechanism of sleep has been thekey interest of the researchers. We have millions of tiny neurons, sending numerous signalsto each other every second. Interestingly, according to research, our brains act very differently when we fall asleep. In particular, specific neural circuits start acting to take control of the behavioral state of sleep-in humans. During sleep, the neuronal signals line up into patterns, more like the waves in a sea, and each wave pattern represents different stages of sleep. When we first lay down trying to

fall asleep, our brain creates fast 'alpha' waves which are associated with mental relaxation. Sequentially, the brain waves slow down producing '*theta*' waves. These theta waves are crucial for learning and memory purposes (Patel et al., 2022). Of note, when we're still not very deeply asleep until we sink into a slow wave of sleep. At this point, the waves are being produced only about once a second. The next interesting thing that happens during the sleep cycle is rapid eye movement or 'REM' sleep (Figure 1). As it sounds, our eyes begin to move very quickly andrandomly behind the eyelids. Importantly, REM sleep is also the stage of sleep when one dreams (Hobson, 2009). Scientists believe dreams happen because the brain sends several signals and tricks our minds to have virtual reality like feelings (Hobson et al., 2014). Interestingly, our brain passes through each of these stages of sleep several times a night like every 90 minutes to 2 hours.

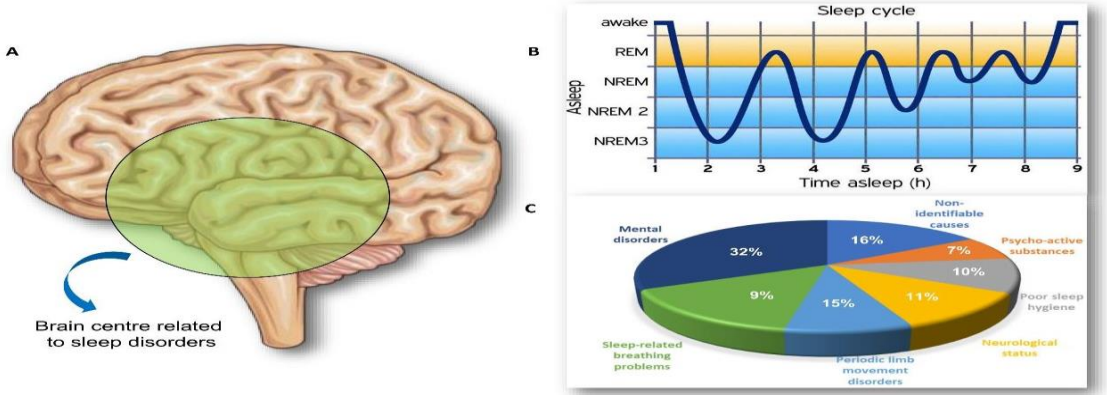


Figure 1: Sleep cycle and the attributes of insomnia

A) The human brain centers on sleep disorders B) Graphical illustration of the normal sleep cycle C) Pie-chart represents the major attributes of insomnia (Data adapted from the published article by Maurice M. Ohayon, Sleep Med Res 2011; 2:1-9).

In 1982, EEG recording by scientists indicated that there are two internally driven processesnamely sleep-dependent (Process S) and sleep-independent circadian (Process C). Process S takes control of sleep promotion whereas Process C controls circadian-mediated wake promotion (Light et al., 2010).

4. Insomnia

Sleep has always been a highly conserved physiological process among humans. Importantly, genes, the circadian rhythm, the external environmental cues, and internal signals from the homeostat play crucial roles in human sleep. We all have this habit of wondering and pondering about our unloved works/issues before sleeping however,chronic disruption of sleep turns into psychiatric disorders, neurodegenerative disorders, andin extreme sleep deprivation, even death (Colten et al., 2006).

4.1 sleep disorders: Who is awake?

We spend approximately one-third of our lives sleeping. Although we don't have clear-cut answers for why we do so or what are the associated inherent mechanisms, 'sleep' is a criticalphysiological mechanism that brings delicate changes in our brain's neuro-physiological activities and is even connected with specific patterns of our sub-consciousness and memory (Alhola and Polo-Kantola, 2007). Therefore, sleep is a unique lack of responsiveness which has some interesting facts and features. The report shows that some disorders are either affected by poor sleep hygiene or the impact of sleep deprivation in significant ways including, obesity, apnea, and insomnia (Beccuti and Pannain, 2011). Insomnia remains one of the most common sleep disorders characterized by difficulty falling asleep, maintaining the state of sleep, or feeling the freshness following a sound sleep. Insomnia is mostly seen in older age groups and is often considered a normal aging phenomenon and is also commonamong adults too. Unending distress (physical and/or emotional), disturbances during sleep(e.g. snoring partner), and unsolvable thinking loops seem to be the trigger factors of insomnia (Levenson et al., 2015). Insomniac people show specific symptoms of fatigue, lessenergy, difficulty in concentrating, mood disturbances, and decreased performance in daily activities. Moreover, the risks of insomnia are known to increase with some medications, drugs, and psychiatric disorders (Roth, 2007).

Interestingly, the sleep deprivations and normal sleep schedule wrecking due to jetlags, sudden workload, or some minor health problems are mostly short-term. Unfortunately, forinsomniacs, the sleepless nights not only keep piling up but also get wracked with anxieties especially so much during bedtime that the stress response system sets the flight or freeze mode (Vargas et al., 2020). The endocrine system comprising hormones like cortisol and adrenocorticotropic hormones are released increasing the heart rate, blood pressure, alertnesstowards nighttime sounds or disturbances, and the overall duration of arousal state. Henceforth, insomnia compromises one's ability to both sleep and rest. (Saddichha, 2010).

4.2 Understanding the mechanism of sleep cycle

Researchers experimentally proved different stages of sleep including, NREM stages (N1, N2, N3) and REM (A. K. Patel et al., 2022). In general, a person goes into a very transient sleep phase called stage N1 before going into stage N2 and eventually into stage N3, where they stay until going into deep REM sleep. Both of the last stages are very physically restful; REM sleep is very mentally restorative and stage N3 is known to producegrowth hormones. After a certain time, the sleep pattern circles back to stage N1 and the above cycle repeats throughout the night. Interestingly, REM sleep gets longer towards the end of the night and stage N3 is usually longer towards the beginning of the sleep. Unfortunately, insomniacs and older persons experience a reduction in both of these types of sleep (D. Patel et al., 2018).

4.3 Types of insomnia

Neuro-psychological experts demonstrated that insomnia can be caused by physical and psychological factors. There is sometimes an underlying medical condition that causes '*chronic acute insomnia*' while '*transient acute insomnia*' may be due to disruptions in circadian rhythm, jet lag, jobs shift changes, high altitudes, environmental noise, extreme heat or cold, psychological issues like bipolar disorder, depression, anxiety or others (Fietzeet al., 2021). In a broad sense, transient acute and chronic acute insomnia lasts less than threemonths and usually resolves on its own without treatment. But, chronic insomnia or insomnia disorder doesn't tend to go away on its own but it does respond well to certain types of treatments and it's almost always caused by negative psychological associations with the bed. Another peculiar type of insomnia is considered adjustment insomnia which is difficulty sleeping associated with a major lifestyle change related to sudden mental stress (Rezaei et al., 2018). It can be typically resolved on its own once we haveadapted to the change in our life. The next type of insomniac pattern is drug-induced insomnia. It can be caused by the long-term use of certain antidepressant medications (Wichniak et al., 2017). Insomnia symptoms are also common as withdrawal from alcohol and cannabis addiction. Comorbid insomnia occurs along with other disorders like depression, cardiovascular disease, asthma, chronic pain, cancer, and arthritis (Khurshid, 2018). Sleep onset insomnia describes a difficulty in falling asleep at the beginning of the night. This type of insomnia is characterized by a delay in the sleep of 30 minutes or more. Another common type is middle insomnia. A person who suffers from middle insomnia maywake up during the night and can't get back to sleep (Krystal et al., 2019). Middle insomniais sometimes associated with excessive alcohol use and chronic pain. Women having middleinsomnia may face hot flashes, and night sweats during menopause can disturb sleep too. Late insomnia attacks can be realized when it feels too early to get up and too late to go backto sleep. Such type of insomnia is often associated with emotional stress, circadian rhythm disorders, and low blood sugar level (Surani et al., 2015). Researchers also observed another insomnia type known as conditioned insomnia; this is when insomnia becomes a conditionedresponse to going to bed (Sharma and Andrade, 2012). Behavioral insomnia in childhood begins when a child under five is not given a strict bedtime. This can cause sleep difficultieslike refusing to go to bed, wanting to sleep with a parent, or getting up repeatedly during the night. This is concerning because untreated behavioral insomnia can cause issues beyond sleepiness like poor performance at school and temper tantrums (Vriend and Corkum, 2011). Idiopathic insomnia describes insomnia with no apparent cause but recent research has found that this type of insomnia probably does have a cause as the hyperactivity of the central nervous system (Momin and Ketvertis, 2022). This leads to an exaggerated response to stressby the body which remains in a hyper-vigilant state making sleep difficult. Interestingly, scientists have found paradoxical insomnia (Rezaie et al., 2018). In this type, sufferers thinkthey spend hours lying awake but the amount of time they spend sleeping is within the normalrange. Finally, we will discuss an important and concerning type of insomnia in recent days.This is considered sleep hygiene insomnia

(Riemann, 2018) which refers to poor habits likeusing electronic gadgets before bed, an inconsistent bedtime, drinking alcohol and coffee before sleep, night-time exposure to bright light from lamps and electronics, etc.

4.4 The adverse effects of insomnia on human body and mind

According to a research, lack of sleep will prevent our brain from being able to initially make new memories (Baena et al., 2020). Therefore, it's almost as though withoutsleep the memory inbox of the brain shuts down. Also, lack of sleep leads to increased production of beta-amyloid in the brain tissue which is associated with Alzheimer's disease (Brzecka et al., 2018). This happens because our bodies eliminate this toxic protein during deep sleep at night, decreasing our risk for developing dementia in later life. Another research finding is very concerning that sleep deprivation affects the reproductive system causing reduced fertility (Lateef and Akintubosun, n.d.). Insomnia causes a feeling of fatigue and weakness throughout the day that severely affects a person's normal sex life. Insomnia is responsible for ruining our body's metabolism severely too. We already know that our brain's primary source of energy is cerebral glucose but during a healthy sleep, our metabolism slows down to conserve this glucose. Current research investigations indicated that the adrenaline hormone that prevents sleep for insomniacs also speeds up the metabolisms thus, burning up the brain's energy giving glucose supply and leaving the insomniac person waking in a state of exhaustion, confusion, and stress (Hirotsu et al., 2015). When these cycles of stress and restlessness last several months, they are diagnosed as chronic insomnia.While insomnia rarely leads to death, its chemical mechanisms are similar to anxiety attacks found in those experiencing depression and anxiety.

5. Therapeutics for insomnia and other sleep-disorders

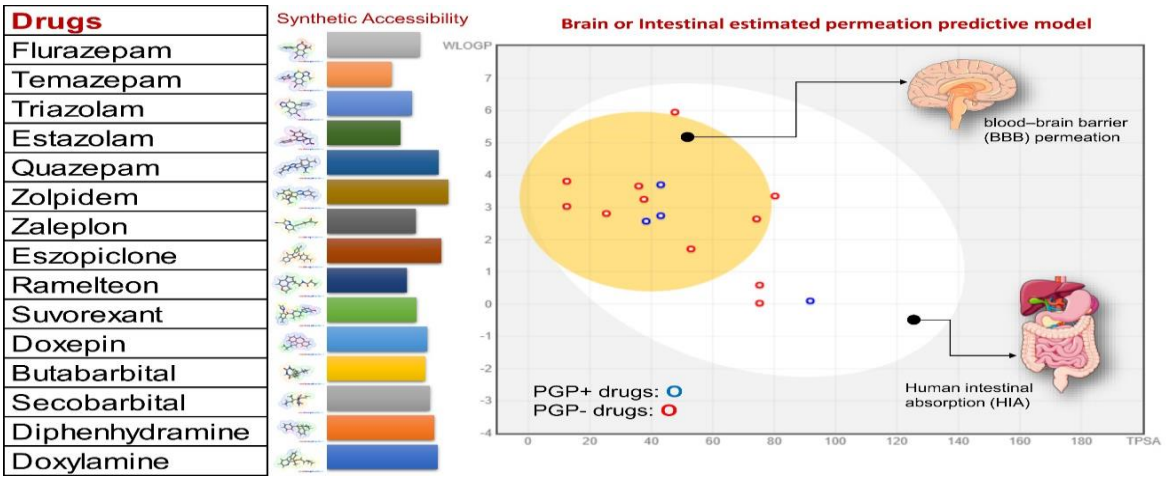


Figure 2: In-Silico prediction of efficacy and bio-availabilities of major insomnia drugs

The diagram represents the synthetic accessibility of major insomnia drugs and categorization of

P-Polyglycoprotein (PGP) positive/negative drugs in brain or Intestinal EstimateD permeation predictive model (BOILED Egg Model). The drug data was taken from the Drug bank server and run in the SwissADME web tool.

Diagnosis of insomnia requires complete comprehensive sleep as well as health histories of the suspected insomniac persons. Clinicians need to recognize and manage the symptoms of insomnia to prevent the morbidity associated with it. Generally, sedative or hypnotic drugs are recommended as a pharmaceutical therapeutic approach for insomnia but many of them have significant toxicities and side effects (Pagel et al., 2018). Even many drugs with addictive potential cause major issues of drug abuse that negatively affect our society (Chakravorty et al., 2018). Notably, it can be challenging to recommend a specific pharmaceutical drug for a patient who complains of insomnia. In recent days, some medicines are identified with very low systemic and organ toxicity, less addictive potential, minimal next-day sleepiness, and low side-effect profile which can be applied safely to treat insomnia (Figure 2). Currently, benzodiazepines (BZDs) are FDA-approved drugs and are allowed to treat insomnia, including triazolam, estazolam, temazepam, quazepam, and flurazepam (Walsh and Mahowald, 1991). Benzodiazepine receptor agonists (BzRAs) include both benzodiazepine (BZD) as well as non-BZD agents. Of note, all of these drugs bind to the gamma- aminobutyric acid (GABA) receptor complex however; they may differ in their affinity to bind in different sites. In particular, non-BZDs called - Z drugs were discovered to minimize the side effects and abuse potential related to BZDs. The report showed that zolpidem, zaleplon, and eszopiclone provided evidence that there was a statistically significant decrease in sleep latency compared with placebo control (Danjou et al., 1999). Zaleplon was found effective as the second non-BZD group of the drug. Its mechanism of action is very quick and it shows its effect in a shorter duration on patients having middle-of-the-night awakenings (Monti et al, 2017). During a clinical trial, both zaleplon and zolpidem showed efficacy for shorteningsleep latency and extending sleep duration. Importantly, Ramelteon was approved for the treatment of insomniac patients who showed difficulty with sleep onset and the drug is a melatonin agonist in nature. Another drug Doxepin is a sedating tricyclic antidepressant that has a high affinity for its tamine receptors (Gillman, 2007). According to a scientific research, CNS depression is achieved with the use of barbiturates which can act in a range of mild sedatives to general anesthesia (Use et al., 2015). Additionally, proper hypnotic doses of these drugs can reduce the sleep latency and the number of awakenings for insomniac patients.

6. Conclusions

Researchers identified the different stages of sleep as NREM stages (N1, N2, N3) and REM (Shirota et al., 2021) where N1 is the transient sleep phase and N2 and N3 are physically restful stages that eventually go into deep and mentally restorative REM sleep. Insomnia is a well-known case of sleep disorder that can be either ‘chronic acute insomnia’ or ‘transient acute insomnia’. Chronic disruption

of sleep may turn into psychiatric disorders, neurodegenerative disorders, and even death in extreme sleep deprivation. Genetic studies on sleep disorders have progressed from twin and family studies to candidate gene approaches to culminate in genome-wide association studies (GWAS) (Raizen and Wu, 2011). Several works disclosed that sleep-wake characteristics, in addition to electroencephalographic (EEG) sleep patterns, have a certain degree of heritability. Notwithstanding, it is rare for sleep disorders to be attributed to single gene defects because of the complexity of the brain network/pathways involved. Besides this, the advancing insights in epigenetic gene-environment interactions add further complexity to understanding the genetic control of sleep and its disorders. Several research studies have indicated that sleep disturbance can impair crucial processes, sometimes leading to chronic pain e.g., joint pain. The future of mechanistic research on sleep and sleep-related pain may target dopamine and opioid systems. Scientists have also found sleep disorders as one of the major risk factors of stroke. Research has proven stroke related sleep disturbances to negatively impact angiogenesis, axonal sprouting, synaptogenesis, ultimately causing aggravate brain damage and hampered neurological recovery.

Medications for managing insomnia should be used for short-term only and non-drug treatment should be considered a priority. Of note, sometimes patients do require multi-modal therapy to recover from insomnia symptoms and improve their quality of life and Z-drugs and anti-depressants are the well-known medication options in most of the cases. However, further research is required towards effective, tailor-made, and individual-specific treatment to achieve success for sleep-disorder and insomnia-affected patients.

7. Acknowledgments

The author is gratefully acknowledges the Institute of Advanced Research, Gandhinagar for providing infrastructure and support.

References

Alhola, P., & Polo-Kantola, P. (2007). Sleep deprivation: Impact on cognitive performance. *Neuropsychiatric Disease and Treatment*, *3* (5), 553–567.

Baena, D., Cantero, J. L., Fuentemilla, L., & Atienza, M. (2020). Weakly encoded memories due to acute sleep restriction can be rescued after one night of recovery sleep.*Scientific Reports*, *10* (1), Article 1. https://doi.org/10.1038/s41598-020-58496-4

Beccuti, G., & Pannain, S. (2011). Sleep and obesity. *Current Opinion in Clinical Nutrition and Metabolic Care*, *14* (4), 402–412. https://doi.org/10.1097/MCO.0b013e3283479109

Brzecka, A., Leszek, J., Ashraf, G. M., Ejma, M., Ávila-Rodriguez, M. F., Yarla, N. S., Tarasov, V. V., Chubarev, V. N., Samsonova, A. N., Barreto, G. E., & Aliev, G. (2018). Sleep Disorders Associated With Alzheimer's disease: A Perspective. *Frontiers in Neuroscience*, *12*, 330. https://doi.org/10.3389/fnins.2018.00330

Bushey, D., Tononi, G., & Cirelli, C. (2011). Sleep and synaptic homeostasis: Structuralevidence in Drosophila. *Science (New York, N.Y.), 332* (6037), 1576–1581. https://doi.org/10.1126/science.1202839

Chakravorty, S., Vandrey, R., He, S., & Stein, M. D. (2018). Sleep Management amongPatients with Substance Use Disorders. *The Medical Clinics of North America*, *102* (4), 733–743. https://doi.org/10.1016/j.mcna.2018.02.012

Cirelli, C., & Tononi, G. (2008). Is Sleep Essential? *PLoS Biology*, *6* (8), e216. https://doi.org/10.1371/journal.pbio.0060216

Colten, H. R., Altevogt, B. M., & Research, I. of M. (US) C. on S. M. and. (2006). Extentand Health Consequences of Chronic Sleep Loss and Sleep Disorders. In *Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem*. National Academies Press (US). https://www.ncbi.nlm.nih.gov/books/NBK19961/

Cross, Z. R., Kohler, M. J., Schlesewsky, M., Gaskell, M. G., & Bornkessel-Schlesewsky, I. (2018). Sleep-Dependent Memory Consolidation and Incremental Sentence Comprehension: Computational Dependencies during Language Learning as Revealed by Neuronal Oscillations. *Frontiers in Human Neuroscience*, *12*, 18. https://doi.org/10.3389/fnhum.2018.00018

Danjou, P., Paty, I., Fruncillo, R., Worthington, P., Unruh, M., Cevallos, W., & Martin, P.(1999). A comparison of the residual effects of zaleplon and zolpidem following administration 5 to 2 h before awakening. *British Journal of Clinical Pharmacology*, *48* (3), 367–374. https://doi.org/10.1046/j.1365-2125.1999.00024.x

Dijk, D.-J., & Landolt, H.-P. (2019). Sleep Physiology, Circadian Rhythms, Waking Performance and the Development of Sleep-Wake Therapeutics. *Handbook of Experimental Pharmacology*, *253*, 441–481. https://doi.org/10.1007/164_2019_243

Fietze, I., Laharnar, N., Koellner, V., & Penzel, T. (2021). The Different Faces of Insomnia. *Frontiers in Psychiatry*, *12*, 683943. https://doi.org/10.3389/fpsyt.2021.683943

Gillman, P. K. (2007). Tricyclic antidepressant pharmacology and therapeutic drug interactions updated. *British Journal of Pharmacology*, *151* (6), 737–748. https://doi.org/10.1038/sj.bjp.0707253

Hirotsu, C., Tufik, S., & Andersen, M. L. (2015). Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. *Sleep Science*, *8* (3), 143–152. https://doi.org/10.1016/j.slsci.2015.09.002

Hobson, J. A. (2009). REM sleep and dreaming: Towards a theory of protoconsciousness.*Nature Reviews. Neuroscience*, *10* (11), 803–813. https://doi.org/10.1038/nrn2716

Hobson, J. A., Hong, C. C.-H., & Friston, K. J. (2014). Virtual reality and consciousness inference in dreaming. *Frontiers in Psychology*, *5*, 1133. https://doi.org/10.3389/fpsyg.2014.01133

Holst, S. C., & Landolt, H.-P. (2015). Sleep Homeostasis, Metabolism, and Adenosine. *Current Sleep*

Medicine Reports, *1* (1), 27–37. https://doi.org/10.1007/s40675-014-0007-3

Kelly, M. L., Collin, S. P., Hemmi, J. M., & Lesku, J. A. (2019). Evidence for Sleep in Sharks and Rays: Behavioural, Physiological, and Evolutionary Considerations. *Brain, Behavior and Evolution*, *94* (1–4), 37–50. https://doi.org/10.1159/000504123

Khurshid, K. A. (2018). Comorbid Insomnia and Psychiatric Disorders: An Update. *Innovations in Clinical Neuroscience*, *15* (3–4), 28–32.

Krystal, A. D., Prather, A. A., & Ashbrook, L. H. (2019). The assessment andmanagement of insomnia: An update. *World Psychiatry*, *18* (3), 337–352. https://doi.org/10.1002/wps.20674

Lateef, O. M., & Akintubosun, M. O. (n.d.). Sleep and Reproductive Health. *Journal ofCircadian Rhythms*, *18*, 1. https://doi.org/10.5334/jcr.190

Levenson, J. C., Kay, D. B., & Buysse, D. J. (2015). The Pathophysiology of Insomnia. Chest, 147 (4), 1179–1192. https://doi.org/10.1378/chest.14-1617

Light, G. A., Williams, L. E., Minow, F., Sprock, J., Rissling, A., Sharp, R., Swerdlow, N.R., & Braff, D. L. (2010). Electroencephalography (EEG) and Event-Related Potentials (ERP's) with Human Participants. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N. Crawley ... [et Al.]*, *CHAPTER*, Unit-6.2524. https://doi.org/10.1002/0471142301.ns0625s52

Momin, R. R., & Ketvertis, K. (2022). Short Term Insomnia. In *StatPearls*. StatPearlsPublishing. http://www.ncbi.nlm.nih.gov/books/NBK554516/

Monti, J. M., Spence, D. W., Buttoo, K., & Pandi-Perumal, S. R. (2017). Zolpidem's usefor insomnia. *Asian Journal of Psychiatry*, *25*, 79–90. https://doi.org/10.1016/j.ajp.2016.10.006

Pagel, J. F., Pandi-Perumal, S. R., & Monti, J. M. (2018). Treating insomnia with medications. *Sleep Science and Practice*, *2* (1), 5. https://doi.org/10.1186/s41606-018-0025-z

Patel, A. K., Reddy, V., & Araujo, J. F. (2022). Physiology, Sleep Stages. In *StatPearls*.StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK526132/

Patel, D., Steinberg, J., & Patel, P. (2018). Insomnia in the Elderly: A Review. *Journal ofClinical Sleep Medicine : JCSM : Official Publication of the American Academy of Sleep Medicine*, *14* (6), 1017–1024. https://doi.org/10.5664/jcsm.7172

Potter, G. D. M., Skene, D. J., Arendt, J., Cade, J. E., Grant, P. J., & Hardie, L. J. (2016).Circadian Rhythm and Sleep Disruption: Causes, Metabolic Consequences, and Countermeasures. *Endocrine Reviews*, *37* (6), 584–608. https://doi.org/10.1210/er.2016-1083

Raizen, D. M., & Wu, M. N. (2011). Genome-Wide Association Studies of Sleep Disorders. *Chest*, *139* (2), 446–452. https://doi.org/10.1378/chest.10-1313 Rattenborg, N. C., Van Der Meij, J., Beckers,

G. J. L., & Lesku, J. A. (2019). LocalAspects of Avian Non-REM and REM sleep. *Frontiers in Neuroscience*, *13*, 567. https://doi.org/10.3389/fnins.2019.00567

Rezaie, L., Fobian, A. D., McCall, W. V., & Khazaie, H. (2018). Paradoxical insomnia and subjective-objective sleep discrepancy: A review. *Sleep Medicine Reviews*, *40*, 196– 202. https://doi.org/10.1016/j.smrv.2018.01.002

Rezaei, M., Khormali, M., Akbarpour, S., Sadeghniiat-Hagighi, K., & Shamsipour, M. (2018). Sleep quality and its association with psychological distress and sleep hygiene: A cross-sectional study among pre-clinical medical students. *Sleep Science*, *11* (4), 274–280.https://doi.org/10.5935/1984-0063.20180043

Riemann, D. (2018). Sleep hygiene, insomnia and mental health. *Journal of SleepResearch*, *27* (1), 3. https://doi.org/10.1111/jsr.12661

Roth, T. (2007). Insomnia: Definition, Prevalence, Etiology, and Consequences. *Journal of Clinical Sleep Medicine : JCSM : Official Publication of the American Academy of SleepMedicine*, *3* (5 Suppl), S7–S10.

Saddichha, S. (2010). Diagnosis and treatment of chronic insomnia. *Annals of Indian Academy of Neurology*, *13* (2), 94–102. https://doi.org/10.4103/0972-2327.64628 Sharma, M. P., & Andrade, C. (2012). Behavioral interventions for insomnia: Theory andpractice. *Indian Journal of Psychiatry*, *54* (4), 359–366. https://doi.org/10.4103/0019- 5545.104825

Shirota, A., Kamimura, M., Kishi, A., Adachi, H., Taniike, M., & Kato, T. (2021). Discrepancies in the Time Course of Sleep Stage Dynamics, Electroencephalographic Activity and Heart Rate Variability over Sleep Cycles in the Adaptation Night in Healthy Young Adults. *Frontiers in Physiology*, *12*, 623401. https://doi.org/10.3389/fphys.2021.623401

Surani, S., Brito, V., Surani, A., & Ghamande, S. (2015). Effect of diabetes mellitus onsleep quality. *World Journal of Diabetes*, *6* (6), 868–873. https://doi.org/10.4239/wjd.v6.i6.868

Use, T., Nakahara, H., Kimoto, A., Beppu, Y., Yoshimura, M., Kojima, T., & Fukano, T. (2015). Barbiturate Induction for the Prevention of Emergence Agitation after Pediatric Sevoflurane Anesthesia. *The Journal of Pediatric Pharmacology and Therapeutics : JPPT*,*20* (5), 385–392. https://doi.org/10.5863/1551-6776-20.5.385

Vargas, I., Nguyen, A. M., Muench, A., Bastien, C. H., Ellis, J. G., & Perlis, M. L. (2020). Acute and Chronic Insomnia: What Has Time and/or Hyperarousal Got to Do with It? *Brain Sciences*, *10* (2), 71. https://doi.org/10.3390/brainsci10020071

Vriend, J., & Corkum, P. (2011). Clinical management of behavioral insomnia of childhood. *Psychology Research and Behavior Management*, *4*, 69–79. https://doi.org/10.2147/PRBM.S14057

Walsh, J. K., & Mahowald, M. W. (1991). Avoiding the blanket approach to insomnia. Targeted therapy for specific causes. *Postgraduate Medicine*, *90* (1), 211–214, 217–219,

223–224. https://doi.org/10.1080/00325481.1991.11700993

Wichniak, A., Wierzbicka, A., Walęcka, M., & Jernajczyk, W. (2017). Effects ofAntidepressants on Sleep. *Current Psychiatry Reports*, *19* (9), 63. https://doi.org/10.1007/s11920-017-0816-4

Review Article

COVID-19: An immunologist's perspective

Shivani Yadav[1], Naveen Challagundla[1], Dhruvi Shah[1], Parameswar Dalai[1], Hima Vora[1], and Reena Agrawal-Rajput[1*]

[1] *Immunology Lab, Institute of Advanced Research (IAR),*

Koba, Institutional Area, Gandhinagar 382426, India

[*] *Correspondence Author: agrawalreena21@gmail.com, reena.rajput@iar.ac.in*

Abstract

An immune response if not mounted properly against any infection might prove havoc ratherthan a help. Corona virus disease 2019 (COVID-19) gained the size of a pandemic in no time. An individual's immunity plays a crucial role in either efficient viral clearance or disease - associated immuno-pathologies. The SARS-CoV-2 infection has shown heterogeneity in its clinical manifestation wherein an optimally directed immunity may be the key to a successful treatment regimen. This may partially be directed by variation in the patient's health and co-morbidities. Any imbalance in immune response might lead to fatalities in addition to pathogen-induced damages. Henceforth, immunomodulatory strategies are of prime importance in the SARS-CoV-2 infections but could vary from patient to patient. This communication briefs about the innate immune responses dictating the manifestation of COVID-19, treatments targeting immune-mechanism in SARS-CoV-2 infections, and a mechanistic overview of immuno-modulatory activities of the major therapeutics exploited to treat this infection.

Keywords: covid-19, innate immunity, inflammation, hydroxychloroquine, azithromycin, remdesivir

1. Introduction

It is well established by now that COVID-19 is caused by a family of SARS-Corona viruseswhich are single-stranded RNA, enveloped viruses, infecting birds and mammals, includinghumans (Rothan and Byrareddy, 2020; Xu et al., 2020). SARS-CoV-2 family demonstrates human to human transmission and is related to the viruses responsible for SARS and MERSoutbreak in previous years. The entire globe is struggling with the disease. COVID-19 is anacute disease, presenting flu-like symptoms in most of the patients along with a loss of tasteand smell, high fever, dry cough, headache, body pain, joint pain, difficulty in breathing, pneumonia, extreme weakness, diarrhea, hemoptysis, nausea, dyspnoea and may extend to metabolic acidosis, septic shock, and bleeding (Rothan and Byrareddy, 2020; Xu et al., 2020) etc. Moreover, recent reports implicate the neurological consequences of SARS-CoV-2 infection too (Needham et al., 2020). The infection can resolve on its

own but can be terminalin some cases with ~3-4% of global mortality rate (WHO, 2020). However, the disease has shown a wide range of heterogeneity in its symptoms and clinical manifestations where someindividuals might show some symptoms like fever, diarrhea, pain, etc. and others might not experience the same and some could even remain asymptomatic. The transmission happens either via direct contact through droplets or through intermediate fomites from symptomatic as well as asymptomatic patients. Preventive measures instituted by the nations worldwide are: social distancing, masks, gloves, hand hygiene practices, case detection, contact tracing, and quarantines. World public health authorities like the Centre for Disease Control and Prevention (CDC), World Health Organization (WHO) and global partners are extending great efforts to circumvent the spread of disease.

To date, a definitive treatment strategy for SARS-CoV-2 relies a lot on clinical symptoms; hence the treatment modalities are directed towards symptomatic treatment and supportive care. However, researchers and clinicians worldwide are working on developing novel drugs,antiviral, vaccines, or repurposing the existing therapies. This review highlights: (i) SARS- CoV-2-induced innate immune pathology; (ii) Treatments targeting immune mechanism in SARS-CoV-2 infections; (iii) Immunomodulatory activities of major drugs are tried as potential therapeutic targets.

2. Innate immune pathology and pathophysiology of SARS-CoV-2

An orchestrated chain of immune events is required which should be efficient enough to clear off the virus from the body and likewise balanced enough not to damage the own body tissues or organs. While in most of the cases of COVID-19, an exaggerated innate immune response to the virus could be considered as one of the culprits for disease associated immune pathology (Cauchois et al., 2020b). The general response against a respiratory infection is aggressive inflammation and results in significant airway damage. Early and late phase COVID-19 patients have been observed to suffer alveolar damage due to hyaline membrane formation, infiltration of mononuclear cells, macrophages in air spaces,and thickening of the alveolar wall (Xu et al., 2020). Multiple organs are affected in biopsy or autopsy reports as indicated by observed spleen atrophies, necrotic lymph nodes, kidney focal hemorrhages, inflammatory cell infiltration, and enlargement of the liver, edema, etc (Rothan and Byrareddy, 2020; Y. Yang et al., 2020). It is hence not only an infection with the virus but also the host mediated responses that contribute to the severity of disease leading to acute respiratory distress syndrome (ARDS). ARDS manifests difficulty in breathing, lowblood oxygen levels, and leads to oxygen unavailability to the tissues (TW et al., n.d.). Thus,ARDS eventually causes respiratory failure that contributes to death in 70% of the fatal COVID-19 cases (TW et al., n.d.; Yang et al., 2020). The pulmonary and systemic inflammation seen in SARS-CoV-2 infections is associated with the innate immune responseto the virus which may further be intensified by the cells of the adaptive immune response (R & S, n.d.). SARS-CoV-2 principally targets via its envelope spike (S) protein- the airway alveolar epithelial cells, vascular endothelium, and macrophages which have abundance of the

receptor angiotensin-converting enzyme 2 (ACE2) (M et al., n.d.). This results in the loss of ACE2 function of regulating the renin-angiotensin system (RAS) that influences blood pressure and electrolyte balance. The outcome contributes to inflammatory response and increased vascular permeability (Channappanavar and Perlman, 2017).

2.1 Pathogenic inflammation associated with SARS-CoV-2 infection

As mentioned above, a hyper inflammation observed during SARS-CoV-2 infections may well add to the pathogenic and clinical manifestations of the disease, further leading to fatalcomplications. Thus, it is of utmost importance to apprehend the source of this pathogenic inflammation in COVID-19 cases and the role of the innate and corresponding adaptive immune response in instigating this hyper inflammation. Past reports with SARS-CoV infections have indicated that the virus can infect the macrophages, dendritic, and the T cellsas well, in addition to the alveolar cells which might be a contributory factor for this aberrantcytokine production even at low viral titers observed in COVID-19 infections (Perlman and Dandekar, 2005; R & S, n.d.). The cell infection is followed by viral replication and releaseinducing pyroptosis and discharge of danger-associated molecular patterns (DAMPs) like nucleic acid, ATP, etc (Perlman and Dandekar, 2005). These released DAMPs are further recognized by neighboring cells that trigger the release of pro-inflammatory cytokines such as Interleukin (IL) -1β, IL-2, IL-7, GCSF, and TNF-α (Stegelmeier et al., 2019). Besides, chemokines like IFN-γ-induced protein 10 (IP10), macrophage inflammatory protein 1α (MIP1α), monocyte chemo-attractant protein-1 (MCP1) recruit monocytes, macrophages, and T cells to the site of infection and enhance the inflammatory response to establish a strong pro-inflammatory feed forward loop termed as **"cytokine storm"** (Dong Kim et al., 2007; Stegelmeier et al., 2019). Many factors contribute to the production of these inflammatory cytokines like hyper activation of innate immune cells, epithelial cell lysis, and activation of inflammatory T cells. Likewise, inflammasome activation in secreting inflammatory cytokines and in studies, SARS-CoV E is reported to play a role in inflammasome activation (Lupfer et al., 2015). Viroporin 3a from SARS-CoV is reported to activate NLPR3 inflammasome (I.-Y. Chen et al., 2019) and ORF3 and ORF8 of SARS- CoV-2 are indicated to cause NLRP3-inflammasome activation (Yuen et al., 2020). Also, elevated circulating $CD4^+$, Th17, and $CD8^+$ cells are observed in the infected patients indicating inflammasome mediated activation of innate immune cells causing IL-1β mediated Th17 differentiation (Xu et al., 2020). Another major cytokine reported to elevatein SARS-CoV-2 patients is IL-6 (Cauchois et al., 2020a; W. Zhang et al., 2020). It was observed to be correlated with the prognosis of patients in a clinical study in Wuhan with 123 patients (W. Zhang et al., 2020). Higher levels of IL-6 show poor prognosis, thus emerging to anti-IL-6 therapies (Wan et al., 2020). Even though reports indicate hypern activation of T cells, lymphopenia is commonly observed during COVID-19 infections and has been suggested as a predictive marker for disease severity (Z. Chen and John Wherry,2020). Lymphocytes also express ACE2, thus possibility is it mediates viral invasion of lymphocytes and lysis (Jurewicz

et al., 2007). Increased pro-inflammatory cytokines: TNF-α and IL-6 observed in COVID-19 patients may also induce lymphocyte apoptosis resultingin lymphopenia (Tan et al., 2020). Additionally, a study in Wuhan reported an increased IL-2R in patients (Zhang et al., 2020). This circulating IL-2R could bind to IL-2 necessary for T cell activation and proliferation, thus decreasing the T cell population as observed in infected patients (Zhang et al., 2020). Thus, these decreased lymphocytes and T cell activation observed in COVID-19 patients could indicate that the source of IL-6 and other cytokines is the activated innate immune cells. Supporting this, Yajing and colleagues in their review suggested that innate immunity might be the source of inflammation along with pyroptosis in macrophages and epithelial cells and IgG-FcR activation in innate immune cells (Fu et al., 2020) (Figure II). Therefore modulating innate immune cells can be an attractive strategyfor COVID-19 treatment (Felsenstein et al., 2020).

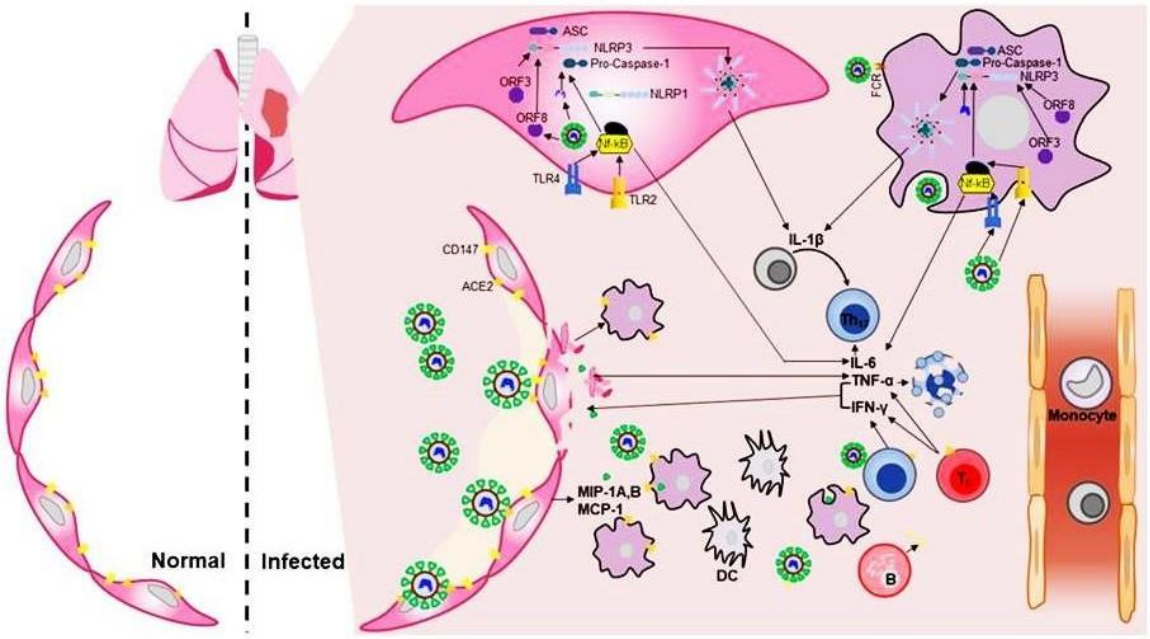


Figure 1: Inflammation during SARS-CoV-2 infection

SARS-CoV-2 infects cells binding to ACE2 and CD147 and replicates in the cells activating NfkB and NLRP3 inflammasome which in turn mediates release of IL-6, TNF-α, and IL-1β respectively. Viruslyses epithelial cells and induces the production of chemokines that recruit macrophage and other immune cells. IL-1β differentiates Naive T cells to pathogenic Th17 which produces IFN-γ. TNF-α, IFN-γ mediate viral inhibition and lysis of infected cells. TNF also induces apoptosis in T cells. These inflammatory cytokines mediate pathogenic cytokine storm in COVID-19.

On the other hand, the critical patients also show elevated levels of anti-inflammatory cytokines such as IL-10 and IL- 4 (Gong et al., n.d.) which is otherwise a rare possibility inresponse to acute viral infection and which might be inducing a possible B cell activity. However, it is perceived that the plasma cells induced thereof may produce non-neutralizingantibodies that might aid in building up the viral burden via antibody dependent enhancement (ADE), further contributing to multi-organ damage (Smatti et al., 2018). Elderly patients and patients with co-morbidity are vulnerable to develop the deregulated immune response and complicated pathophysiology that fails to limit the virus while kids and youngsters, despite high viral titers tend to display better protection (Raoult et al., 2020). With an increasing evidence of inflammation in SARS-COV-2 patients, the scientific community has split into two parts suggesting immunosuppressive treatment to decrease inflammation and others suggest that reducing inflammation could impair pathogen clearance (Zhang et al., 2020).

A healthy host with a functional immune response would limit viral load either by preventingits entry or fusion with the host cell. The inflammatory immune cells would attract virus specific T cells that would clear off the infected cells and prevent surrounding cells from virus infection, thus preventing lung damage (Huber et al., 2014). However, during the SARS-CoV-2 infection, early damage to the alveolar epithelial cells and macrophages trigger a local immune response (Guillon et al., 2020). The local immune mediators recruitother innate immune cells which later prime and recruit protective T and B cells. SARS- CoV-2 is sensitive to type I interferon-induced clearance which also induces IFN-stimulatedgenes (ISGs) that are important inducers of early antiviral immunity (Oh et al., 2012). The T cells via IFN-g limit the spread of the virus by the lytic effect on the virally infected cells, consequently preventing viral dissemination to the other cells. Additionally, the B cells maygenerate neutralizing antibodies to neutralize the virus particles which can be further cleared off by phagocytosis (Tay et al., 2020). Thus, a host equipped with a functional immune response would clear the virus with minimum recovery time which happens in the majorityof healthy individuals infected with SARS-CoV-2 (Channappanavar et al., 2014).

Following the entry into host cells, the virus encounters innate immune cells that facilitate their clearance. However, to build up infection, the virus must evade innate immunity (Prompetchara et al., 2020). Also, deviation from the above mentioned immune activation and viral clearance would

lead to improper, unbalanced immune response and can end up incomplications risking the patient's life. The mechanism of evasion for SARS-CoV-2 is not worked upon however, there are lessons learned from SARS-CoV infection. Even infectionwith SARS-CoV-2 demonstrates a deregulated immune response in older and co-morbid patients where the host fails to mount the desired immunity and a dysfunctional immune response occurs (di Mauro Gabriella et al., 2020). The innate immune cells may drive a burstof the inflammatory response which may in addition to its effect on viruses and infected cells, result in a lytic effect on the uninfected cells. The establishment of infection in the hostprogresses and may cause severe lung damage and even have systemic effects.

Immune cell localization to the lungs justifies lymphopenia and increased neutrophil- lymphocyte ratio during SARS-CoV-2 infection in almost 80% of the patients. Uncontrolled leukocyte infiltration damages pulmonary cells via excessive secretion of proteases and reactive oxygen species leading to desquamation of alveolar cells and pulmonary edema that limits gas exchange and poor availability of oxygen to the tissues. The infected tissues get prone to secondary infections which also contribute to the production of excessive cytokines.This cytokine storm worsens pulmonary inflammation and causes acute lung damage and issignificantly higher in patients under critical care. These excessive cytokines circulate to the other organs of the patient leading to multi-organ damage and subsequent failure in severe conditions (Wong et al., 2004).

3. Treatments targeting immune mechanism in SARS-CoV-2 infections

Some of the possible therapies, particularly those directed towardsmanipulating the innate immune response leading to hyper-inflammation have been summarized.

3.1 Immunosuppressives

In severe SARS-CoV-2 infected patients, antivirals progressively decrease viral load but there is still a noteworthy mortality rate which indicates the failure of antivirals in COVID-19 treatment. Thus, immune-compelled damages could be held accountable for the observed mortality. In the critical cases of COVID-19 where patients become severely unwell because of the life-threatening hyper inflammatory state, they may experience multiple organ failure including respiratory failure and eventually death of the patient. Timely recognition of inflammation and apt initiation of immunosuppression may benefit severely ill COVID-19 patients.

3.1.1 Antirheumatic drugs; IL-37and IL-38

Antirheumatic drugs are broadly immunosuppressive and anti-inflammatory. Various anti- rheumatic drugs like chloroquine and hydroxychloroquine, azithromycin, IL-6 inhibitors, IL-1β inhibitors are being explored for their beneficial effects in COVID-19 patients (Georgiev,2020). SARS-CoV-2 binds to the Toll-Like Receptor (TLR) mediating inflammasome activation. The active caspase-1 further cleaves pro-IL-1β to mature IL-1β which facilitateslung inflammation, fever, and fibrosis

(Freeman and Swartz, 2020). Thus, it is a logical therapeutic approach to suppress the inflammasome activation of pro-inflammatory cytokines like IL-1 family members and IL-6 in inflammatory diseases including viral infections like COVID-19.

IL-37 is an immunosuppressive cytokine. It exerts its effects in its pro-forms, extracellularly.Pro-IL-37 is cleaved to active IL-37 by caspase-1 and binds to SMAD3 to act as a transcription factor for anti-inflammatory genes as SMAD3-IL-37 complex (Yan et al., 2019). It is reported to suppress both innate and acquired immune responses. IL-37 is also reported to inhibit inflammation via its ability to act on IL-18α receptor (Wang et al., 2018). IL-37 is known to inhibit macrophage differentiation, activation, antigen presentation,and secretion of inflammatory cytokines like IL-6, TNF-α, and IL-1β by inhibiting MyD88,NF-κB, and ICAM-1 and activating AMP kinase (Wang et al., 2018; Xie et al., 2016; Yanet al., 2019). Epithelial cell derived IL-37 is reported to inhibit both T cell and DCs activation in the inflammatory mucosa of inflammatory bowel disease (Caraffa et al., 2018). Furthermore, it is reported by McNamee and colleagues that transgenic expression of human IL-37 greatlyprotects the mouse against LPS-induced shock (McNamee et al., 2011). Thus, the subdual of IL-1β by IL-37 in the COVID-19 induced hyper inflammatory state can prove a new therapeutic strategy.

Similarly, IL-38 is the latest cytokine of the IL-1 family produced by macrophages and B cells. It inhibits IL-1β and other pro-inflammatory IL-family cytokines (Mora et al., 2016). It is reported to inhibit the activation of the intracellular STAT1, STAT3, p38 MAPK, ERK1/2, and NF-κB pathways (Sun et al., 2020). IL-38 has been reported to confer protection against sepsis by boosting the immunosuppressive activities of $CD4^+$ $CD25^+$ Tregcells (Ge et al., 2020). This could be of use in inducing tissue protective and homeostatic immunity in critical COVID-19 cases facing fatalities due to hyper inflammation and cytokine storm. Perricone et al. (2020) have proposed anti-rheumatic drugs to be repurposedfor SARS-CoV-2 infection. Colchicine was highlighted in the article for its activity via inflammasome inhibition. Other cytokine neutralization strategies may also demonstrate success against COVID-19. A novel adjunctive therapy is Cytosorb which is being tested against SARS-CoV-2. It treats cytokine storm in critically ill sepsis or cardiac surgery patients. A broad spectrum of cytokines, DAMPs, and PAMPs are all absorbed by cytosorb, which lowers their circulating levels and alleviates disease outcomes (Tay et al., 2020).

3.1.2 IL-1 Antagonists

Acute inflammation can be fatal as observed in many inflammatory disorders, sepsis, allergic reactions, and SARS-CoV-2 infections. As already discussed, a hyper-activated inflammatory response could lead to multiple organ failure and death. SARS-CoV-2 infections are no exception to this. Thus, blocking the inflammatory mediators could be oneof the promising strategies to combat the fatal outcomes of the disease. One of the key cytokines mediating the inflammatory responses is the IL-1. IL-1 could also be called the chief cytokine of local and systemic inflammation and the pathological role of IL-1 facilitated inflammation has been observed in several diseases (Dinarello

et al., 2012). Anakinra is one of the safest established IL-1 antagonists and has a short half life along withmultiple routes of administration, making it an attractive anti-inflammatory therapeutic in critical cases of COVID-19. It is a recombinant IL-1 receptor antagonist (IL-1Ra) and is known to block the activity of both IL-1α and IL-1β. Anakinra has already been used as a therapeutic in various inflammatory diseases like rheumatoid arthritis, gout, sepsis, type 2 diabetes, etc (Dinarello et al., 2012). Other than that, neutralization of IL-1 with antibodies like canakinumab (anti-IL-1β neutralizing monoclonal antibody) can be done. Other therapeutic approaches including IL-1α neutralization and orally active small molecule inhibitors of IL-1 production, such as caspase-1 inhibitors could also be explored for treatinghyper-inflammation in COVID-19 cases. In a recent Lancet report, Omar Maoujoud and colleagues have advocated the use of Anakinra in combination with autophagy activators for attenuatingthe cytokine storm in SARS-CoV-2 infections (Maoujoud et al., 2020). Similarly, a cohort study published in The Lancet concluded that the use of Anakinra leads to a decreased requisite for invasive ventilation support and reduced mortality in critically ill COVID-19 patients without any major side-effects (*Anakinra for Severe Forms of COVID-19: a Cohort Study - the Lancet Rheumatology*, n.d.). The study found that when a group of 52 patients were administered with Anakinra subcutaneously (100 mg twice daily for 72 h, then 100 mgdaily for 7 days), the observed mortality and ICU admission rate for ventilation support was reduced to 25% in Anakinra group as compared to observed rate of 73% in control group receiving no Anakinra. Another study by Raphaël Cauchois and colleagues published in PNAS supports the use of Anakinra for preventing hyper-inflammation and cytokine storm in critically ill COVID-19 patients for avoiding complications and respiratory failure. Likewise, a 300 mg, subcutaneous administration of Canakinumab was found to be safe, effective, well tolerated, and resulted in a fast decline of systemic inflammation along with improved oxygenation in ten hospitalized patients of COVID-19 with pneumonia, hyper- inflammation, and respiratory failure (Ucciferri et al., 2020). Thus, it can be concluded that the early blockade of the IL-1 receptor could be a successful therapeutic approach for treating acute hyper inflammation and associated respiratory failure in COVID-19 cases.

3.1.3 IL-6 Antagonists

IL-6 is a soluble cytokine that is rapidly secreted in response to injuries, tissue damages, infections, etc. and exerts its effect via prompting quick acute phase responses, inflammatory immune reactions, and haematopoiesis (Tanaka et al., 2014). IL-6 is known to exert a varietyof immunological functions or biological activities, e.g., IL-6 is known as B-cell stimulatoryfactor 2 (BSF-2) because of its ability to differentiate activated B cells into antibody producing plasma cells and hepatocyte-stimulating factor (HSF) because of its effect on acute-phase protein synthesis by hepatocytes. Most importantly, IL-6 is also known as interferon (IFN)-β2 due to its antiviral activity (Tanaka et al., 2014). It plays a vital role in initiating inflammatory immune responses against infections and shows antiviral activities. Thus, dysregulation and a persistent synthesis will trigger pathological effects and tissue

damage dueto chronic inflammation (Liu et al., 2017; Tanaka et al., 2014). In critical cases of COVID- 19, a check on excessive IL-6 production could be a helpful strategy to avoid disease related immune pathologies. Tocilizumab is a humanized, anti-IL-6 receptor antibody that has been clinically tested in various trials and has shown its efficacy for controlling chronic inflammation and thus, is approved for the treatment of rheumatoid and juvenile idiopathic arthritis (Tilg et al., 1997). Therefore, it is highly likely that Tocilizumab is anticipated to be helpful in other immune facilitated diseases like end stage cases of COVID-19 and respiratory failure due to alveolar injury. Thus, IL-6 antagonists like Tocilizumab and Sarilumab are underway for their efficacy against SARS-CoV-2 and can prove beneficial in combination with other drugs. IL-6 antagonism inhibits inflammatory T cell ($CD4^+$Th1 and$CD4^+$Th17) differentiation and increases co-inhibitory molecule PD1-PDL1, and effectivelyactivates $CD8^+$ T cells in chronic infections. Thus, IL-6 antagonism inhibits inflammation and increases effective $CD8^+$ T cell activation needed for viral clearance (Velazquez-Salinas et al., 2019).

3.1.4 Corticosteroids

Corticosteroids are established immunosuppressives known for their anti-inflammatory properties (Barshes et al., 2004). They are commonly used for the treatment of acute and chronic inflammations like rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis, etc. They exert their effect by suppressing inflammatory pathways in innate immune cells like co-oxygenase or transrepression of NF-κB and AP-1, the known inflammatory regulators (Barshes et al., 2004; Coutinho and Chapman, 2011). Because of theiranti-inflammatory properties, corticosteroids might help in easing the COVID-19 associatedsystemic and local inflammation and tissue damage. But, their use in treating COVID-19 patients should be meticulously evaluated as at one side steroids might lend a helping hand in saving critically ill patients from immune-compelled fatalities whereas on the other side, steroid administration might end up making patients more susceptible to secondary bacterial infections and pro-longed viral clearance. Past studies with SARS-CoV-1 and MERS-CoV and COVID-19 treatment trials with inhaled corticosteroids indicate that corticosteroids didnot improve viral clearance and lung functions instead led to secondary infections (Tang etal., 2020; Veronese et al., 2020). Both WHO and CDC advise against the use of corticosteroids in COVID-19. Contrarily, the Randomized Evaluation of COVID-19 therapy (RECOVERY Trial) conducted in patients with severe COVID-19 requiring oxygen therapy or ventilator support, has shown a considerably better disease outcome withthe use of dexamethasone- a corticosteroid (Horby et al., 2020) (NCT04381936). Besides, corticosteroids have mainly been considered in COVID-19 to avoid the much discussed cytokine storm which eventually leads to complications like ARDS, anaphylactic shock, multiple organ failure, and death. Various reports indicate that cytokine storm occurs in the second week of COVID-19 (Ragab et al., 2020), thus preferably a corticosteroid therapy should be tried in this period or by the 6-8 days of infection to prevent the advancement of disease towards

cytokine storm and associated fatalities. As corticosteroid treatment in COVID-19 is considered controversial (Veronese et al., 2020), it is crucial to decide at what stage of COVID-19 progression and up to what extent the corticosteroid treatment should be given to save a critical ill patient's life. Therefore, from an immunological pointof view, the time of administration of corticosteroid therapy would play a defining role in apositive or a negative disease outcome.

3.1.5 PPARγ Agonists

Peroxisome proliferator activated receptor (PPARs) are a family of ligand activated nuclearhormone receptors and PPARγ agonists like Thiazolidinediones (TZDs) - Pioglitazone and rosiglitazone are commonly used for treating insulin resistance that accompanies with amplified inflammation (Chiarelli and Di Marzio, 2008). PPAR- γ are highly expressed in adipose tissues but are also found in several other cell types like airway smooth muscle,epithelial cells, macrophages, etc. PPAR- γ agonists are reported to inhibit the release of inflammatory factors (Chiarelli and Di Marzio, 2008). PPAR-γ is known to induce apoptosis, thus shifting the inflammatory machinery by inhibiting NF-kB to apoptotic pathways (Kytikova et al., 2020). It has been well reported to inhibit pro-inflammatory cytokines release from activated macrophages and airway epithelial cells and promote apoptosis in endothelial cells, vascular smooth muscle cells, T- lymphocytes, macrophages, etc (Birrell et al., 2004). Pioglitazone and rosiglitazone have also been reported to act via interfering with monocyte chemo-attractant protein 1 and its monocyte receptor (CCR2), thus preventing immune cell recruitment and thereby inflammation (Chiarelli and Di Marzio, 2008). PPARγ agonists were observed to reduce IL-8 and MMP-9 release from airway epithelial cells *in vitro* and reduced airway inflammation in mice (Perez et al., 2008). Thus, in COVID-19 cases, the use of PPARγ agonist TZDs like Pioglitazone and Rosiglitazone might help via their ability to weaken the excessive inflammatory immune response as evident in atherosclerosis and cysticfibrosis model of infection. TZDs may help limit the local inflammation and associated lungdamage when prescribed at the right stage of COVID-19 disease progression (Ciavarella et al., 2020).

3.1.6 Redox-active pro-drugs

The metronidazole is a nitroimidazole, anti-protozoal, and anti-bacterial redox-active pro- drug that acts as a biocide via interaction with a nitroreductase (Ciavarella et al., 2020). Metronidazole is investigated for its immunomodulatory properties in addition to its anti- microbial nature (Shakir et al., 2011). Metronidazole is reported to substantially reduce the percentage of circulating neutrophils, PBMCs, and monocytes with simultaneous increasein circulating lymphocytes (Ciavarella et al., 2020). The study also reported a marked suppression in splenocytes and human peripheral blood lymphocyte proliferation on metronidazole treatment (Ciavarella et al., 2020). Furthermore, the study observed that metronidazole administration leads to a decline in delayed-type hypersensitivity (DTH) reaction, phagocytic, and TNF-α secretion by peritoneal macrophages evidencing the immunosuppressive nature of metronidazole. Metronidazole is known to decrease the levels of

neutrophil generated reactive oxygen species during inflammation and several pro- inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α, IL12, IL1α, IFN-γ, and C-reactive protein(CRP) levels and neutrophil count which are known to be increased during the COVID-19 infection (Shakir et al., 2011). Thus, attributing to the anti-inflammatory properties of metronidazole, it could be explored as a potential drug target to curb the cytokine storm- related immuno-pathologies in critical COVID-19 cases (Gharebaghi et al., 2020).

3.1.7 Mesenchymal stem cell transplantation

Mesenchymal stem cells (MSCs) are known for their wide-ranging, hearty immunomodulatory functions. MSCs are multipotent cells that can differentiate into a variety of mesenchymal lineage cells like adipocytes, osteocytes, chondrocytes, etc (Kaplan et al., 2012). The MSCs are also reported to migrate and participate in repair of the areas of tissue injury (M. Kaplan et al., 2012). Interestingly, MSCs have also been investigated for their anti-inflammatory and immunomodulatory properties (Kaplan et al., 2012; Yi and Song, 2012). It has been found that MSCs can inhibit T and B cell proliferation, reduce cytotoxic T (CTL), and NK cell activity (Yi and Song, 2012) which could be of use in critical cases of COVID-19 where excessive CTL and NK cell activity could lead to alveolar damage and lung tissue scarring/damage, leading to respiratory failure.MSCs are also been reported to negatively affect and obstruct the maturation and antigen- presenting activities of DCs along with modulating the macrophage function (Le Blanc and Ringdén, 2007; P. De Miguel et al., 2012). Additionally, *in vivo* MSCs administration in models of autoimmune disease like arthritis, autoimmune diabetes, etc. has already evidenced the much observed immune regulatory roles of MSCs (P. De Miguel et al., 2012).Thus, MSCs can be considered as a potential immune modulating strategy in controlling hyper activated immune reactions in COVID-19 cases which often lead to disease associatedimmune pathologies and associated fatalities. Moreover, a study including 7 patients explored these immunomodulatory impacts of MSCs in patients manifesting COVID-19 associated pneumonia. A million MSCs/ kilogram bodyweight of patients was given and a noteworthy clinical improvement in all patients was observed (Leng et al., 2020).

3.2 Convalescent plasma

High titers of neutralizing immunoglobulin containing plasma from recovered COVID-19 patients are fundamentally used in this therapy to provide passive antibody protection againstviral infection which was found effective in previous SARS infections. Reduction in nasopharyngeal SARS-CoV-2 load leading to better oxygenation after plasma therapy has been observed (Kong et al., 2020). However, there are both positive and negative reports inthe case of this therapy (Sullivan and Roback, 2020) as antibodies in the plasma can mediate increased uptake of the virus via FcR mediated phagocytosis.

3.3 Vaccines

The development of a successful vaccine is one of the most awaited treatment strategies forCOVID-

19. Vaccination is the most rapid and economical strategy to combat SARS-CoV-2infection. Vaccine strategies can be based on virus like particles, subunit components, RNA,DNA, proteins/peptides, and attenuated viruses. The most effective vaccines should elicit long-term antigen specific antibody producing memory B cells. During SARS-CoV-2 infection, both humoral and cellular immune responses play a crucial role in eradicating the virus. To clear the infection, the vaccine should also elicit both humoral and cellular immunity. DNA/RNA vectors work similarly to the virus. These vectors express target protein in the cytoplasm and require highly efficient carriers. Subunit vaccines of S protein of SARS-CoV-2 induce antibody production and $CD8^{+}$T cell response. The antibodies produced in the S protein subunit vaccine can also act as neutralizing antibodies preventingviral entry. They are very safe vaccine candidates and less prone to side effects and induce an appropriate immune response when combined with a suitable adjuvant. Attenuated vaccines by gene deletion of viral replication essential proteins are in preclinical stages. The immune response generated is very strong against viruses and persists for a longer time as the viral components for both humoral and cytotoxic T cells exist. However, there is a risk of reversion to a virulent form. Whole killed viruses are also the better and rapid strategy to produce a vaccine which can generate multiple antibodies against the virus. In most of the cases, heat-killed viruses induce hyper sensitivity which might be a problem in SARS-CoV-2 infected patients. The vaccines, however, should not only focus on generating plasma cells,they should be able to generate cytotoxic T cells, to efficiently kill virus-infected cells, thuspreventing further virus spread (Pandey et al., 2020). Many of the pharmaceutical companiesand educational institutes are trying the above strategies to fast track vaccine production and many are in various stages of Phase-I and Phase-II clinical trials. Vaccine research is not a sprint race, it is a marathon and thus it might take 12-18 months till the vaccine reaches globally. Controlled human trials may fast track the process but, it has its limitations.

Table I: Clinical trial phases for covid19 vaccines

According to https://covid19.trackvaccines.org, there are 40 approved vaccines in 197 countries with 11 WHO EUL vaccines. Further, there are 220 vaccine candidates with 753 vaccine trials in 78 countries. Table I indicates the status of current vaccine trial and their status:

Sr. No.	Phase	Numbers
1.	Approved	40
2.	Phase III trials	84
3.	Phase II trials	71
4.	Phase I trials	56

3.4 Vitamin C and D

For COVID-19 treatment, doctors have been recommending vitamin C and vitamin D supplements along with the prescribed antivirals and antibacterials. Low vitamin C levels are reported in patients with infections, due to metabolic changes (Hemilä, 2017; Marik, 2018). Vitamin C is evidenced for its anti-inflammatory and anti-oxidant properties (Carr andMaggini, 2017). It has already been reported that vitamin C increases natural killer cell proliferation and lessens the ROS (reactive oxygen species) production which is one of thecontributory factors for the activation of inflammasome (Huijskens et al., 2015).

Consequently, it affects the secretion of pro-inflammatory cytokines- IL1β and IL-18 which are involved in inflammatory systemic syndrome or cytokine storm (Canali et al., 2014). Vitamin C is known to block ICAM-1 expression and NFkB activation (Hung et al., 2008).Thus, Vitamin C affects the development and proliferation of lymphocytes along with their functions, e.g., it has been reported to promote the development and proliferation of T cells (Kouakanou et al., 2020). It has been reported that melanoma primed DCs when pre-incubated with vitamin C and then co-cultured with T cells, resulted in an increased killingof melanoma cells and improved generation of effector CD8 T-cells (Jeong et al., 2014). Furthermore, vitamin C has been reported to reduce the incidence of pneumonia in the common cold in various studies (Sasazuki, 2016). Furthermore, a study result by Paul E Marik and colleagues suggest that if intravenous vitamin C is used in the early stages of disease progression along with corticosteroids and thiamine (Hydrocortisone, Vitamin C, and Thiamine- HAT therapy), positive improvements are observed in patients' health via effective prevention of progressive organ dysfunction. Hence, a decreased mortality in patients with severe sepsis and septic shock was observed (Marik et al., 2017). On the same line, a clinical trial (NCT01434121) study result suggests that intravenous administration of ascorbic acid infusion was safe and well-tolerated in patients with lessened progression of patients towards multiple organ failure and decreased biomarkers of inflammation and decreased endothelial injury in lungs (Fowler et al., 2014). The study which included 24 severe sepsis patients who were randomized in 1:1:1 to receive intravenous infusions of vitamin C/ six hours for four days at low (50 mg/kg/24 h, n = 8) or high (200 mg/kg/24 h, n = 8)

doses of ascorbic acid or placebo (5% dextrose/water, n = 8) reported that ascorbic acid infusion resulted in quick and considerable increase in plasma ascorbic acid levels with no side effects. Ascorbic acid infusions resulted in a significant reduction in pro-inflammatory biomarker C-reactive protein (CRP) and pro-calcitonin as compared to placebo group. Also, the reduced thrombomodulin levels in ascorbic acid infused patients as compared to placebo group indicated decreased vascular endothelial injury. Thus, vitamin C administration may prove a promising therapeutic in preventing the SARS-CoV-2 infection towards a sever sepsis and multiple organ failure stage, however the time of administration of vitamin C can be a crucial deciding factor.

Vitamin D dearth in individuals has been known an ally to augmented infection episodes. Vitamin D is involved in the production of antimicrobial peptides like cathelicidinand strongly up regulates antimicrobial peptide gene expression (*CAMP*- humans possess this single cathelicidin gene) and is reported for its anti-inflammatory properties (Gombart, 2009). An association between vitamin D deficiency and SARS-CoV-2 infection andmortality has already been reported (Kenneth Weir et al., 2020). Low levels of vitamin D arefound to be associated with an increase in inflammatory cytokines. One of the studies on adults has reported that there is an inverse relationship between the serum levels of 25- hydroxyvitamin D and TNF-α an inflammatory cytokine (De Vita et al., 2014) and the major one of the culprit in inducing cytokine storm and septic shock. Vitamin D deficiency is foundto be related to increased levels of IL-6 (Ansemant et al., 2013; Manion et al., 2017). Thus, these studies are reporting clear indications that vitamin D levels are related to the productionof inflammatory cytokines like TNF-α and IL-6 and vitamin D seems to down regulate the production of these cytokines. These findings seem to validate the notion that a healthy diet with antioxidants, vitamins like C and D, along with the prescribed medications could be of utmost importance in maintaining a balanced immune response to clear off the infection efficiently. These studies also indicate the possibility that vitamin D and C may lessen the occurrence of cytokine storm observed in COVID-19. Furthermore, the role of regulatory Tcells could not be neglected in controlling the inflammation induced and CTL induced tissue damage against a viral infection (D'Alessio et al., 2009). As there is a reported surge in inflammatory damage and cytokine storm in lungs leading to ARDS in COVID-19 cases, itcould be related to the low levels of Treg reported in COVID-19 patients and strikingly lower in severely ill patients (G. Chen et al., 2020). This is also supported by the fact that high levels of Treg are reported to be associated with decreased incidences of respiratory viral disease (Christiaansen et al., 2016). In a nutshell, these observations in various studies relating tothe Treg levels and severity of respiratory viral disease suggest that the increased Treg levelswould be possibly beneficial in lessening the severity of COVID-19 induced ARDS and respiratory failure if mounted aptly (Stephen-Victor et al., 2020). Now it is of interest to know that vitamin D is reported to increase the Treg levels (Fisher et al., 2019).

Of note, it is also reported that COVID-19 patients face thrombotic complications (Avila et al., 2020;

Yang et al., 2020) and vitamin D is reported to play a role in the regulation of thrombotic pathways (Targher et al., 2012). It is important to consider that thrombotic episode and vitamin D deficiency are positively related i.e., if there is an increase in vitaminD deficiency, the thrombotic episodes are also reported to increase. To support the possiblerole of vitamin D and COVID-19 severity, different studies have been conducted (Kenneth Weir et al., 2020). One of the studies reported a noteworthy relationship between the numberof SARS-CoV-2 infections, mortality, and vitamin D levels. It was reported that the most vulnerable population towards SARS-CoV-2 infection is the one which is the most deficient invitamin D (Ilie et al., 2020). Thus, vitamin C and D supplements along with recommended drugs could be a plausible therapeutic approach to resolve SARS-CoV-2 infection effectively.

3.5 ACE2 blockers and protease inhibitors

SARS-CoV2 enters the cells via binding its S protein to ACE2 on host cells expressed on epithelial, endothelial, and immune cells. Blocking virus entry can be an attractive strategy against COVID-19. *In silico* analysis has predicted a Janus kinase (JAK) inhibitor, baricitinib, to inhibit ACE2 (Cava et al., 2020). Similarly, another JAK inhibitor, ruxolitinib,is being tested in clinical trials in China for the treatment of COVID-19 (Cao et al., 2020). Supplementation with soluble ACE2 may also induce viral neutralization and the strategy is currently in clinical trials by APEIRON**.** Alternatively, monoclonal antibodies targeting ACE2 may interfere with viral entry and/or fusion. Similarly, the trans-membrane proteaseserine 2 (TMPRSS2) that cleaves the S protein of the SARS-CoV-2 may be targeted with inhibitors like Nafamostat mesylate and Camostat mesylate. Camostat mesylate tested on isolated SARS- CoV-2 prevented viral entry into lung cells (Pandey et al., 2020). In anutshell, these strategies can be used in combination with the above mentioned immunomodulatory approaches to effectively control COVID-19 disease progression in critical, co-morbid, and andold-aged patients.

4. Molecular insights into immunomodulatory actions of the experimental antiviral treatments tested for SARS-CoV-2

In a need to find the therapies to combat SARS-CoV-2, small clinical trials without control groups have become common. This gives very minimum time to study the molecular mechanism and mode of action and possible systemic manipulation by the drug. An open- label, non-randomized study was carried out which suggested that a combination of hydroxychloroquine, an antimalarial drug and azithromycin, an antibiotic may be beneficial for treating patients with severe COVID-19. Hydroxychloroquine reduced viral load in COVID-19 patients via its immunomodulatory activity while azithromycin reinforces the effects of hydroxychloroquine (Thorlund et al., 2020). The authors have discussed that the synergy shown by azithromycin could be through the prevention of bacterial super infections.However, a few more facts regarding the possible role of azithromycinin this setting would be brainstormed. In synergy to Gautret and colleagues study, Henning Ulrich & Micheli M.

Pillat has indicated that azithromycin may be inhibiting the CD147 on the host cells which also interacts with the S protein of SARS-CoV-2 virus. This may also prevent the dissemination of the virus to other cells including stem cells, thus decreasing infection (Ulrichand Pillat, 2020).

An effort is undertaken to elucidate possible molecular mechanisms reported earlier for the drugs which are highly used in SARS-CoV-2 infections, to make possible sense in disease relief (Figure 2).

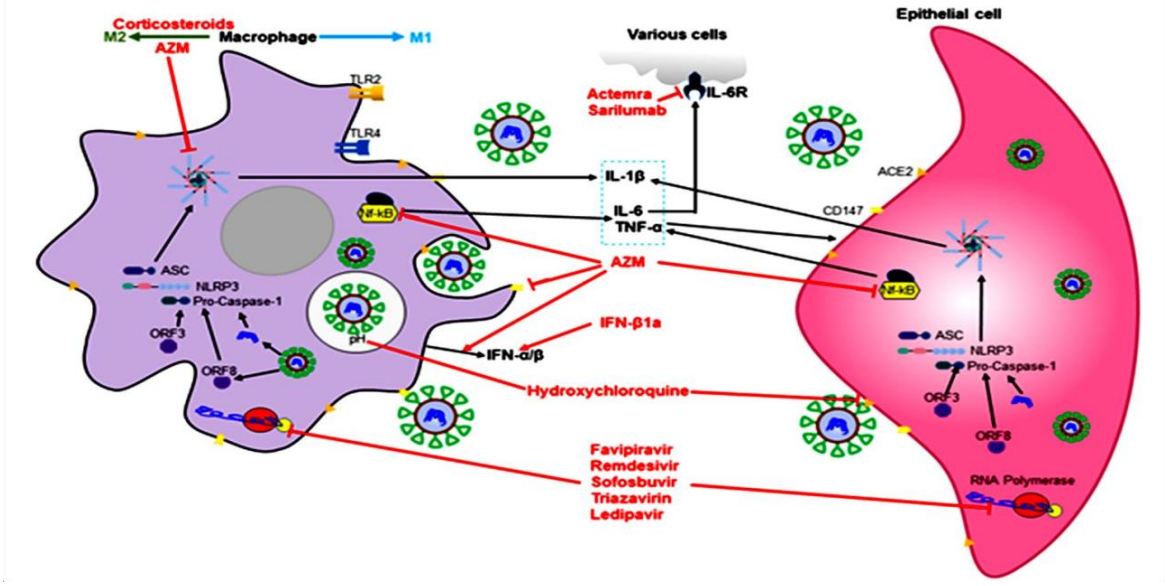


Figure 2: Proposed mechanism of anti-covid-19 drugs

SARS-CoV-2 infection induces cytokine storm indicated in the blue dotted box. Proposed drugs are indicated in red and their mechanisms of inhibition were elucidated.

4.1 Hydroxychloroquine

The medical uses of chloroquine and hydroxychloroquine as anti-malarial drugs date back to1995. Due to its immune-modulatory properties, it is used for treating auto-immune disorders such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), antiphospholipid syndrome (APS), etc. and even in graft rejection (Gilman et al., 2000). Hydroxychloroquinehas been explored as an anti-retroviral drug in HIV and Dengue (Gilman et al., 2000). Chloroquine/Hydroxychloroquine exhibits antiviral effects against SARS-CoV-2 infection via various mechanisms. As stated above, SARS-CoV-2 binds to the ACE2 for its entry into the host cells. Vincent and colleagues have observed that pre-treatment of chloroquine, restricted the entry of SARS-Cov-2 by interfering with terminal glycosylation of ACE2 that negatively affects the virus receptor binding and limits its infection (Vincent et al., 2005). Recently, Jacques Fantini and colleagues have revealed that hydroxychloroquine competitively binds to the ganglioside such that viral S protein obstructs its contact with ACE-2 receptor, thus hinderingviral entry in host cells (Fantini et al., 2020).

In COVID-19 infections, hyper activation of innate immunity induces additional tissue damage

leading to ARDS. In antigen presenting cells, hydroxychloroquine interferes with TLR7 ligand binding and inhibits subsequent inflammasome activation and triggering of DNA sensors like cGAS-STING, thus reducing the release of pro-inflammatory cytokines mainly IL-6 and IL-1β that play an important role in a cytokine storm. Moreover, hydroxychloroquine polarizes macrophages to anti-inflammatory phenotype (Shiratori et al., 2018) which might help in reducing inflammation, thus relieving the pathogenesis of SARS-CoV-2.

Initially, it was believed that hydroxychloroquine accumulation in lysosomes helped to alleviate SARS-CoV-2 viral insufficiency by causing a pH shift from acidic to basic, which rendered the lysosomal enzymes inactive. One of the mechanisms that contribute to COVID-19 pathology is the persistent activation of T cells due to the presence of viral antigens that driveT cells to a non-responsive state known as T cell exhaustion (Chen and Wherry, 2020).The lysosomal pH modulation property of chloroquine also prevents MHC-II mediated antigen presentation and contributes to immunemodulatory effects. Thus, hydroxychloroquine via decreased antigen presentation inhibits T cell exhaustion. Besides, adaptive immunity is essential for restricting viral infections. However, during the hyperactivation state, the release of T cell-dependent cytokine and cellular cytotoxicity contributes to tissue inflammation and viral persistence. Hydroxychloroquine inhibits the expression of CD154 (CD40L) which is quite mandatory for T cell activation. Thus, hydroxychloroquine limits the effector T cell responses which in turn curbs dysregulation inthe adaptive immune response. Moreover, Yang and colleagues have reported that hydroxychloroquine prevents Th17 cell differentiation and IL-17 production that are known to be elevated during SARS-CoV-2 infection (Yang et al., 2018).

The clinical trials by Chen and colleagues have suggested a promising efficacy of hydroxychloroquine in COVID-19 patients (Chen et al., 2020). Contrarily, some of the observations show no rapid clearance of the SARS-CoV-2 virus and increased mortality ratein COVID-19 infected patients (Magagnoli et al., 2020). The current reports are of small clinical trials with varying patient parameters. Thus, the efficacy of the drug varies with a varied clinical background; however, overall published reports indicate that hydroxychloroquine is quite helpful in the early stage of the disease but not in severe conditions. Detailed molecular exploration with defined patient sub-grouping will further elucidate the role of hydroxychloroquine in SARS-CoV-2 infection.

4.2 Azithromycin

Azithromycin is a known broad spectrum macrolide antibiotic, which binds to the 50S ribosomal sub-unit of bacteria and thus works by inhibiting the bacterial protein synthesis (Williams and Berkley, 2018). Apart from its antibacterial effect, it is well established as an immunomodulatory drug and is known to exhibit anti-inflammatory effects (Ianaro et al., 2000). However, the antiviral

activities of azithromycin have not been explored much. However, in the recent outbreak of COVID-19, azithromycin has been used in combination with other drugs like hydroxychloroquine for possible treatment of the disease (Rosendaal, 2020). Clinical trials conducted in France suggested that azithromycin along with hydroxychloroquine decreased viral load in COVID-19 patients (Rosendaal, 2020). Still, the mechanism of action by which azithromycin could aid in possible antiviral defense is not clear.

Drug	Proposed targets	Mode of action	Reference
Hydroxychloroquine	ACE2 glycosylation	Changes the PH of endosome and post-entry events Inhibits terminal glycosylation of ACE2 decreasing viral binding	Savorino A et al., 2006; Wang M, et al., 2020; Vincent MJ et al., 2005; Borba MGS et al., 2020
Favipiravir	RNA dependent RNA polymerase	Inhibits the RNA-dependent RNA polymerase	Delang L et a., 2018; Eloy P et al., 2020; Du YX et a., 2020
Lopinavir/Ritonavir	Viral proteases	Inhibits viral growth	Vanden Eyden JJ et al., 2020; Chu CM et al., 2004; Choudhury S et al., 2020.
Remdesivir	RNA polymerase	Inhibits the viral application	Agostini ML et a., 2018; Mahase et al., 2020; Yin W et a., 2020.
Umifenovir	Fusion inhibitor	Inhibits fusion between viral and cellular membrane	Vigant F et., 2015; Sattler B et al., 2020
Actemra or tocilizumab	IL-6 Receptor	Decreases inflammatory burden of host	Fu B eta., 2020
Sarilumab	Anti-IL-6R monoclonal antibody	Decreases inflammatory burden of host	Zhang J et a., 2020
Sofosbuvir/Galidesivir	RNA dependent RNA polymerase (RdRp)	Anti-RNA dependent RNA polymerase (RdRp)	Elfiky et al., 2020
Oseltamivir	Inhibits the neuraminidase enzyme	Blocks release of virus from infected cell	Costanzo M et a., 2020
Triazavirin	Guanine nucleotide analog	Prevents viral replication	Luo M Et ., 2013
ledipasvir	Anti-Non-structural protein 5A (NS5A)	Prevents viral RNA replication	Chen YW et al., 2020
Baloxavir Marboxil	Cap-endonuclease inhibitor	antiviral polymerase complex	Hayden FG et al., 2018
Barcitinib	JAK1 and JAK2	Reduces cytokine release	Richardson P et al., 2020
Methylprednisolone	Glucocorticoid	Suppresses immune activation	Arabi YM et a., 2018;

Table 2: Mechanism of action of drugs tested for SARS-CoV-2

The positive effects of azithromycin might be due to its cribbing of pathogenic inflammationassociated with COVID-19 cases. Earlier, Banjanac and colleagues have reported the anti- inflammatory effects of azithromycin on macrophages. Azithromycin interactions with $cPLA_2$ trigger inadequate translocation of the enzyme decreasing eicosanoid production, and eventually affecting the macrophage biological membranes. The drug was shown to inhibit the synthesis of all eicosanoids produced downstream of COX and inhibited arachidonic acid release in mouse macrophages (Banjanac et al., 2012). Furthermore, azithromycin has been reported to have a negative effect on NF-κB activation and synthesisof pro-inflammatory cytokines in various reports (Haydar et al., 2019a). It is also reported to polarize macrophages towards alternatively activated anti-inflammatory M2 like phenotype (Haydar et al., 2019a). The authors attributed this M2 polarizing effect of azithromycin to its ability to alter NF-κB (Haydar et al., 2019a) and STAT1 signaling pathways via decreased p65 nuclear translocation and IKKβ kinase activity (Haydar et al., 2019b). Moreover, in vivo, indication of pulmonary anti-inflammatory activity of azithromycin has already been reported which is attributed to the inhibition of NFκB activation in the lungs (Stellari et al.,2014). Besides, azithromycin has been reported to have its effect on the inflammasome/IL- 1β axis, where it inhibited the secretion of IL-1α and IL-1β in human monocytes and subdued the induction of caspase-4 (Gualdoni et al., 2015). The study suggested that azithromycin might be a drug of choice for treating diseases having deregulated inflammasome activation like acute pulmonary respiratory infections leading to ARDS etc.

Also, azithromycin is reported to show its effect on NK cells and DCs other than macrophages which could be a plausible reason for its immunomodulatory properties. Syh-Jae Lin and colleagues demonstrated that azithromycin down regulated the NK cell cytotoxicity *in vitro,* possibly through down regulating perforin expression and reduced the IFN-gamma and TNF-α production from NK-92 cells (Lin et al., 2012). Azithromycin significantly increased IL-10 production in DCs and modulated the expression of co-stimulatory molecules- CD80 and CD40 (Sugiyama et al., 2007). Since, NKs and DCs both play a major role in orchestrating the immune responses against viral infections, the possible reported effects of azithromycin on these cells should be considered in the expected role of azithromycin in COVID-19 treatment. Lessons from earlier studies in various infections/diseases indicate that it modulates innate immune response by inhibiting inflammatory output and might promote an anti-inflammatory phenotype, which might help in reducing the cytokine storm and in turn help in reducing pathology of SARS-CoV-2. However, pathways involved in inflammation play an essential part in the clearance of microbes as well. Further studies are required to judge whether the immune-modulating properties of azithromycin reported in various studies could lead to a favorable disease outcome in cases like COVID-19.

Apart from its antibacterial and anti-inflammatory effects, various time to time studies have reported probable antiviral activities of azithromycin. Azithromycin was observed as a promising

drug candidate for Anti-Zika Virus activity in Human Glial Cells (Saiz and Martín-Acebes, 2017). Aline Schögler and colleagues in 2015 showed that azithromycin could be a potentialantiviral drug candidate for the treatment of virus associated pulmonary exacerbations caused by rhinoviruses (RVs) which contribute to cystic fibrosis (CF) morbidity. The study reported azithromycin elevated PRRs, thus inducing type I, type III IFNs, and ISGs thereby reducing RV1B replication in CF bronchial epithelial cells (Schögler et al., 2015). However, azithromycin was inefficient in reducing RV1B induced pro-inflammatory cytokines IL-8 and IL-6 levels *in vitro* as opposed to its established anti-inflammatory properties. With COVID-19 clinical reports and earlier understanding of the mechanism of action of azithromycin, it might be possible that it shows its positive effects by decreasing inflammation and promoting antiviral innate immunity.

4.3 Antivirals

Antivirals like Remdesivir, a pro-drug to adenosine was recently cleared for use in SARS- CoV-2 infection in the USA. It has been used for other viral infections like Ebola and Marburg. Its efficacy against corona viruses has been reported earlier (Agostini et al., 2018).It inhibits RNA polymerase competing with ATP and substituting for adenosine during RNAsynthesis (Agostini et al., 2018). During the SARS-CoV-2 outbreak in Wuhan, Wuhan Virus Research Institute has reported Remdesivir to be inhibiting viral infection in cell cultures.

M.L Holshue and colleagues reported the first successful treatment of SARS-CoV-2 treatment with remdesivir (Wang et al., 2020). Gilead Sciences is also sponsoring the study to assess the potential of remdesivir. Apart from antiviral, it has been reported to be anti-inflammatory inmetabolic syndrome related systemic inflammation. It is reported to inhibit NfkB and STING, indicating that remdesivir can act as anti-inflammatory, thus helping SARS-CoV-2 patients (Chang et al., 2020). Although remdesivir is a broad-spectrum antiviral, its efficacyagainst other viruses cannot be generalized to SARS-CoV-2.

In months of COVID-19 pandemic, numerous clinical trials of various sizes and combinations including antivirals, immuno-modulators, plasma therapy, and unproven cell therapies have emerged across the globe. In the wake of many clinical trials, authors present a consolidated mechanism of action of drugs tested in various countries for SARS-CoV-2 (Table 2).

5. Conclusion and remarks

The dysregulation of innate immunity plays an important role in the pathophysiology of COVID-19. The comorbidity-prone patients exhibit hyper-activation of innate immunity, which mediates the cytokine storm that determines the outcomes of the disease. Hence, it is important to balance the immune toxicity and immune suppression to combat COVID-19 infections which can be done by targeting the innate immunity and associated responses along with hampering viral replication. The fact that different treatment approaches may be necessary for different patients, depending on their individual clinical manifestations, should therefore be considered of utmost importance.

Acknowledgment

SY's fellowship is provided by ICMR-Senior Research Fellowship (SRF). NC is funded by ICMR-SRF. DS is supported by the Lady Tata Memorial Jr.Scholarship. PD and HV are funded by the SHODH fellowship by DST, Govt. of Gujarat.

References

Agostini, M. L., Andres, E. L., Sims, A. C., Graham, R. L., Sheahan, T. P., Lu, X., Smith, E. C., Case, J. B., Feng, J. Y., Jordan, R., Ray, A. S., Cihlar, T., Siegel, D., Mackman, R. L.,Clarke, M. O., Baric, R. S., & Denison, M. R. (2018). Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *MBio*, *9* (2), 1–15. https://doi.org/10.1128/mBio.00221-18

Anakinra for severe forms of COVID-19: A cohort study—The Lancet Rheumatology. (n.d.). Retrieved September 5, 2020, from https://www.thelancet.com/journals/lanrhe/article/PIIS2665-9913 (20)30164-8/fulltext Ansemant, T., Mahy, S., Piroth, C., Ornetti, P., Ewing, S., Guilland, J. C., Croisier, D., Duvillard, L., Chavanet, P., Maillefert, J. F., & Piroth, L. (2013). Severe hypovitaminosis D correlates with increased inflammatory markers in HIV infected patients. BMC Infectious Diseases, 13 (1). https://doi.org/10.1186/1471-2334-13-7

Avila, J., Long, B., Holladay, D., & Gottlieb, M. (2020). Thrombotic complications of COVID-19. In American Journal of Emergency Medicine. W.B. Saunders. https://doi.org/10.1016/j.ajem.2020.09.065

Banjanac, M., Munić Kos, V., Nujić, K., Vrančić, M., Belamarić, D., Crnković, S., Hlevnjak, M., & Eraković Haber, V. (2012). Anti-inflammatory mechanism of action of azithromycin in LPS-stimulated J774A.1 cells. Pharmacological Research, 66 (4), 357–362. https://doi.org/10.1016/j.phrs.2012.06.011

Barshes, N. R., Goodpastor, S. E., & Goss, J. A. (2004). Pharmacologic immunosuppression. In Frontiers in Bioscience (Vol. 9, pp. 411–420). Frontiers in Bioscience. https://doi.org/10.2741/1249

Birrell, M. A., Patel, H. J., McCluskie, K., Wong, S., Leonard, T., Yacoub, M. H., & Belvisi, M. G. (2004). PPAR-γ agonists as therapy for diseases involving airway neutrophilia. European Respiratory Journal, 24 (1), 18–23. https://doi.org/10.1183/09031936.04.00098303

Canali, R., Natarelli, L., Leoni, G., Azzini, E., Comitato, R., Sancak, O., Barella, L., & Virgili, F. (2014). Vitamin C supplementation modulates gene expression in peripheral blood mononuclear cells specifically upon an inflammatory stimulus: A pilot study in healthy subjects. Genes and Nutrition, 9 (3), 1–13. https://doi.org/10.1007/s12263-014- 0390-x

Cao, Y., Wei, J., Zou, L., Jiang, T., Wang, G., Chen, L., Huang, L., Meng, F., Huang, L., Wang, N., Zhou, X., Luo, H., Mao, Z., Chen, X., Xie, J., Liu, J., Cheng, H., Zhao, J., Huang, G., … Zhou, J.(2020). Ruxolitinib in treatment of severe coronavirus disease 2019 (COVID-19): A multicenter, single-blind, randomized controlled trial. Journal of Allergy and Clinical Immunology, 146 (1), 137-146.e3. https://doi.org/10.1016/j.jaci.2020.05.019

Caraffa, A., Conti, C., D'Ovidio, C., Gallenga, C. E., Tettamanti, L., Mastrangelo, F.,Ronconi, G., Kritas, S. K., & Conti, P. (2018). New concepts in neuroinflammation: Mast cells pro-inflammatory and anti-inflammatory cytokine mediators. In *Journal of BiologicalRegulators and Homeostatic Agents* (Vol. 32, Issue 3, pp. 449–454). Biolife s.a.s.

Carr, A. C., & Maggini, S. (2017). Vitamin C and immune function. In *Nutrients* (Vol. 9,Issue 11). MDPI AG. https://doi.org/10.3390/nu9111211

Cauchois, R., Koubi, M., Delarbre, D., Manet, C., Carvelli, J., Blasco, V. B., Jean, R.,Fouche, L., Bornet, C., Pauly, V., Mazodier, K., Pestre, V., Jarrot, P.-A., Dinarello, C. A., & Kaplanski, G. (2020a). Correction for "Early IL-1 receptor blockade in severe inflam- matory respiratory failure complicating COVID-19," by. *Proc. Natl. Acad. Sci. U.S.A*, *117*, 18951–18953. https://doi.org/10.1073/pnas.2009017117

Cauchois, R., Koubi, M., Delarbre, D., Manet, C., Carvelli, J., Blasco, V. B., Jean, R., Fouche, L., Bornet, C., Pauly, V., Mazodier, K., Pestre, V., Jarrot, P.-A., Dinarello, C. A., &Kaplanski, G. (2020b). Early IL-1 receptor blockade in severe inflammatory respiratory failure complicating COVID-19. *Proceedings of the National Academy of Sciences of the United States of America*, *117* (32), 18951–18953. https://doi.org/10.1073/pnas.2009017117

Cava, C., Bertoli, G., & Castiglioni, I. (2020). In silico discovery of candidate drugs againstcovid-19. *Viruses*, *12* (4), 1–14. https://doi.org/10.3390/v12040404

Chang, W. T., Liu, P. Y., Gao, Z. H., Lee, S. W., Lee, W. K., & Wu, S. N. (2020). Evidencefor the Effectiveness of Remdesivir (GS-5734), a Nucleoside-Analog Antiviral Drug in theInhibition of I K (M) or I K (DR) and in the Stimulation of I MEP. *Frontiers inPharmacology*, *11* (July), 1–15. https://doi.org/10.3389/fphar.2020.01091

Channappanavar, R., & Perlman, S. (2017). Pathogenic human coronavirus infections: Causes and consequences of cytokine storm and immunopathology. In Seminars in Immunopathology (Vol. 39, Issue 5, pp. 529–539). Springer Verlag. https://doi.org/10.1007/s00281-017-0629-x

Channappanavar, R., Zhao, J., & Perlman, S. (2014). T cell-mediated immune response to respiratory coronaviruses. In *Immunologic Research* (Vol. 59, Issues 1–3, pp. 118–128). Humana Press Inc. https://doi.org/10.1007/s12026-014-8534-z

Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, H., Wang, T., Zhang, X., Chen, H.,Yu, H., Zhang, X., Zhang, M., Wu, S., Song, J., Chen, T., Han, M., Li, S., Luo, X., Zhao, J.,& Ning, Q. (2020). Clinical and immunological features of severe and moderate coronavirusdisease 2019. *Journal of Clinical Investigation*, *130* (5), 2620–2629. https://doi.org/10.1172/JCI137244

Chen, I.-Y., Moriyama, M., Chang, M.-F., & Ichinohe, T. (2019). Severe Acute RespiratorySyndrome Coronavirus Viroporin 3a Activates the NLRP3 Inflammasome. *Frontiers in Microbiology*, *10* (JAN), 50. https://doi.org/10.3389/fmicb.2019.00050

Chen, Z., Hu, J., Zhang, Z., Jiang, S., Han, S., Yan, D., Zhuang, R., Hu, B., & Zhang, Z. (2020). *Efficacy of hydroxychloroquine in patients with COVID-19: Results of a randomizedclinical trial*. *7*. https://doi.org/10.1101/2020.03.22.20040758

Chen, Z., & John Wherry, E. (2020). T cell responses in patients with COVID-19. In *NatureReviews Immunology* (Vol. 20, Issue 9, pp. 529–536). Nature Research. https://doi.org/10.1038/s41577-020-0402-6

Chiarelli, F., & Di Marzio, D. (2008). Peroxisome proliferator-activated receptor-γ agonistsand diabetes: Current evidence and future perspectives. In *Vascular Health and RiskManagement* (Vol. 4, Issue 2, pp. 297–304). Vasc Health Risk Manag. https://doi.org/10.2147/vhrm.s993

Christiaansen, A. F., Syed, M. A., Ten Eyck, P. P., Hartwig, S. M., Durairaj, L., Kamath, S.S., & Varga, S. M. (2016). *Altered Treg and cytokine responses in RSV-infected infants*. https://doi.org/10.1038/pr.2016.130

Ciavarella, C., Motta, I., Valente, S., & Pasquinelli, G. (2020). Pharmacological (or synthetic) and nutritional agonists of PPAR-γ as candidates for cytokine storm modulation in covid-19 disease. In *Molecules* (Vol. 25, Issue 9). MDPI AG. https://doi.org/10.3390/molecules25092076

Coutinho, A. E., & Chapman, K. E. (2011). The anti-inflammatory and immunosuppressiveeffects of glucocorticoids, recent developments and mechanistic insights. In *Molecular and Cellular Endocrinology* (Vol. 335, Issue 1, pp. 2–13). Mol Cell Endocrinol. https://doi.org/10.1016/j.mce.2010.04.005

D'Alessio, F. R., Tsushima, K., Aggarwal, N. R., West, E. E., Willett, M. H., Britos, M. F., Pipeling, M. R., Brower, R. G., Tuder, R. M., McDyer, J. F., & King, L. S. (2009). CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *Journal of Clinical Investigation*, *119* (10), 2898–2913. https://doi.org/10.1172/JCI36498

De Vita, F., Lauretani, F., Bauer, J., Bautmans, I., Shardell, M., Cherubini, A., Bondi, G., Zuliani, G., Bandinelli, S., Pedrazzoni, M., Dall'Aglio, E., Ceda, G. P., & Maggio, M. (2014). Relationship between vitamin D and inflammatory markers in older individuals. *Age*, *36* (4). https://doi.org/10.1007/s11357-014-9694-4

Di Gabriella M., Cristina, S., Concetta, R., Francesco, R., & Annalisa, C. (2020). SARS- Cov-2 infection: Response of human immune system and possible implications for the rapid test and treatment. International Immunopharmacology, 84. https://doi.org/10.1016/j.intimp.2020.106519

Dinarello, C. A., Simon, A., & Van Der Meer, J. W. M. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. In *Nature Reviews Drug Discovery*(Vol. 11, Issue 8, pp. 633–652). Nat Rev Drug Discov. https://doi.org/10.1038/nrd3800

Dong Kim, K., Zhao, J., Auh, S., Yang, X., Du, P., Tang, H., & Fu, Y. X. (2007). Adaptiveimmune cells temper initial innate responses. *Nature Medicine*, *13* (10), 1248–1252. https://doi.org/10.1038/nm1633

Fantini, J., Di Scala, C., Chahinian, H., & Yahi, N. (2020). Structural and molecular modelling studies reveal a new mechanism of action of chloroquine and hydroxychloroquine against SARS-CoV-2 infection. *International Journal of Antimicrobial Agents*, *55* (5), 105960. https://doi.org/10.1016/j.ijantimicag.2020.105960

Felsenstein, S., Herbert, J. A., McNamara, P. S., & Hedrich, C. M. (2020). COVID-19: Immunology and treatment options. In *Clinical Immunology* (Vol. 215). Academic Press Inc. https://doi.org/10.1016/j.clim.2020.108448

Fisher, S. A., Rahimzadeh, M., Brierley, C., Gration, B., Doree, C., Kimber, C. E., Cajide, A. P., Lamikanra, A. A., & Roberts, D. J. (2019). The role of Vitamin D in increasing circulating T regulatory cell numbers and modulating T regulatory cell phenotypes in patients with inflammatory disease or in healthy volunteers: A systematic review. PLoS ONE, 14 (9). https://doi.org/10.1371/journal.pone.0222313

Fowler, A. A., Syed, A. A., Knowlson, S., Sculthorpe, R., Farthing, D., DeWilde, C., Farthing, C. A., Larus, T. L., Martin, E., Brophy, D. F., Gupta, S., Fisher, B. J., & Natarajan,
R. (2014). Phase I safety trial of intravenous ascorbic acid in patients with severe sepsis.*Journal of Translational Medicine, 12* (1). https://doi.org/10.1186/1479-5876-12-32

Freeman, T. L., & Swartz, T. H. (2020). Targeting the NLRP3 Inflammasome in Severe COVID-19. In Frontiers in Immunology (Vol. 11, p. 1518). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2020.01518

Fu, Y., Cheng, Y., & Wu, Y. (2020). Understanding SARS-CoV-2-Mediated Inflammatory Responses: From Mechanisms to Potential Therapeutic Tools. In *Virologica Sinica* (Vol. 35, Issue 3, pp. 266–271). Science Press. https://doi.org/10.1007/s12250-020-00207-4

Ge, Y., Huang, M., Wu, Y., Dong, N., & Yao, Y. (2020). Interleukin- 38 protects against sepsis by augmenting immunosuppressive activity of $CD4^+$ $CD25^+$ regulatory T cells. *Journal of Cellular and Molecular Medicine, 24* (2), 2027–2039. https://doi.org/10.1111/jcmm.14902

Georgiev, T. (2020). Coronavirus disease 2019 (COVID-19) and anti-rheumatic drugs. In Rheumatology International (Vol. 40, Issue 5, pp. 825–826). Springer. https://doi.org/10.1007/s00296-020-04570-z

Gharebaghi, R., Heidary, F., Moradi, M., & Parvizi, M. (2020). Metronidazole a Potential Novel Addition to the COVID-19 Treatment Regimen. *SSRN Electronic Journal*, *8* (1), 40. https://doi.org/10.2139/ssrn.3559020

Gilman, A. L., Chan, K. W., Mogul, M., Morris, C., Goldman, F. D., Boyer, M., Cirenza, E., Mazumder, A., Gehan, E., Cahill, R., Frankel, S., & Schultz, K. (2000). Hydroxychloroquine for the Treatment of Chronic Graft-Versus-Host Disease. *Biology of Blood and Marrow Transplantation*, *6* (3 A), 327–334. https://doi.org/10.1016/S1083-8791
(00)70058-9

Gombart, A. F. (2009). The vitamin D-antimicrobial peptide pathway and its role in protection against infection. In Future Microbiology (Vol. 4, Issue 9, pp. 1151–1165). NIH Public Access. https://doi.org/10.2217/fmb.09.87

Gong, J., Dong, H., Xia, Q., Huang, Z., Wang, D., Zhao, Y., Liu, W., Tu, S., Zhang, M., Wang, Q., & Lu, F. (n.d.). *Correlation Analysis Between Disease Severity and Inflammation-related Parameters in Patients with COVID-19 Pneumonia*. https://doi.org/10.1101/2020.02.25.20025643

Gualdoni, G. A., Lingscheid, T., Schmetterer, K. G., Hennig, A., Steinberger, P., & Zlabinger, G. J. (2015). Azithromycin inhibits IL-1 secretion and non-canonical inflammasome activation. *Scientific Reports*, *5*. https://doi.org/10.1038/srep12016

Guillon, A., Hiemstra, P. S., & Si-Tahar, M. (2020). Pulmonary immune responses againstSARS-CoV-2 infection: Harmful or not? *Intensive Care Medicine*, 1–4. https://doi.org/10.1007/s00134-020-06170-8

Haydar, D., Cory, T. J., Birket, S. E., Murphy, B. S., Pennypacker, K. R., Sinai, A. P., & Feola, D. J. (2019a). Azithromycin Polarizes Macrophages to an M2 Phenotype via Inhibition of the STAT1 and NF-κB Signaling Pathways. *The Journal of Immunology*, *203* (4), 1021–1030. https://doi.org/10.4049/jimmunol.1801228

Haydar, D., Cory, T. J., Birket, S. E., Murphy, B. S., Pennypacker, K. R., Sinai, A. P., & Feola, D. J. (2019b). Azithromycin Polarizes Macrophages to an M2 Phenotype via Inhibition of the STAT1 and NF-κB Signaling Pathways. *The Journal of Immunology*, *203* (4), 1021–1030. https://doi.org/10.4049/jimmunol.1801228

Hemilä, H. (2017). Vitamin C and infections. In *Nutrients* (Vol. 9, Issue 4). MDPI AG. https://doi.org/10.3390/nu9040339

Huber, S. R., van Beek, J., de Jonge, J., Luytjes, W., & van Baarle, D. (2014). T cell responses to viral infections - Opportunities for peptide vaccination. In Frontiers in Immunology (Vol. 5, Issue APR).
Frontiers Research Foundation. https://doi.org/10.3389/fimmu.2014.00171

Horby, P., Lim, W. S., Emberson, J., Mafham, M., Bell, J., Linsell, L., Staplin, N., Brightling, C., Ustianowski, A., Elmahi, E., Prudon, B., Green, C., Felton, T., Chadwick, D., Rege, K., Fegan, C., Chappell, L. C., Faust, S. N., Jaki, T., … Group, R. C. (2020). Dexamethasone for COVID-19-Preliminary Report Effect of Dexamethasone in Hospitalized Patients with COVID-19-Preliminary Report RECOVERY Collaborative Group*. MedRxiv, 2020.06.22.20137273.

https://doi.org/10.1101/2020.06.22.20137273

Huijskens, M. J. A. J., Walczak, M., Sarkar, S., Atrafi, F., Senden-Gijsbers, B. L. M. G., Tilanus, M. G. J., Bos, G. M. J., Wieten, L., & Germeraad, W. T. V. (2015). Ascorbic acid promotes proliferation of natural killer cell populations' in culture systems applicable for natural killer cell therapy. *Cytotherapy*, *17* (5), 613–620. https://doi.org/10.1016/j.jcyt.2015.01.004

Hung, C. F., Huang, T. F., Chen, B. H., Shieh, J. M., Wu, P. H., & Wu, W. Bin. (2008). Lycopene inhibits TNF-α-induced endothelial ICAM-1 expression and monocyte- endothelial adhesion. European Journal of Pharmacology, 586 (1–3), 275–282. https://doi.org/10.1016/j.ejphar.2008.03.001

Ianaro, A., Ialenti, A., Maffia, P., Sautebin, L., Rombolà, L., Carnuccio, R., Iuvone, T., D'Acquisto, F., & Di Rosa, M. (2000). Anti-inflammatory activity of macrolide antibiotics. *The Journal of Pharmacology and Experimental Therapeutics*, *292* (1), 156–163.

Ilie, P. C., Stefanescu, S., & Smith, L. (2020). The role of vitamin D in the prevention of coronavirus disease 2019 infection and mortality. *Aging Clinical and Experimental Research*, *32* (7), 1195–1198. https://doi.org/10.1007/s40520-020-01570-8

Jeong, Y.-J., Kim, J.-H., Hong, J.-M., Seung Kang, J., Kim, H.-R., Jae Lee, W., & Hwang, Y. (2014). Vitamin C treatment of mouse bone marrow-derived dendritic cells enhanced CD8 + memory T cell production capacity of these cells in vivo. Immunobiology, 219, 554– 564. https://doi.org/10.1016/j.imbio.2014.03.006

Jurewicz, M., McDermott, D. H., Sechler, J. M., Tinckam, K., Takakura, A., Carpenter, C. B., Milford, E., & Abdi, R. (2007). Human T and natural killer cells possess a functional renin-angiotensin system: Further mechanisms of angiotensin II-induced inflammation. *Journal of the American Society of Nephrology*, *18* (4), 1093–1102. https://doi.org/10.1681/ASN.2006070707

Kenneth Weir, E., Thenappan, T., Bhargava, M., & Chen, Y. (2020). Does Vitamin D deficiency increase the severity of COVID-19? *Clinical Medicine, Journal of the Royal College of Physicians of London*, *20* (4), E107–E108. https://doi.org/10.7861/CLINMED.2020-0301

Kong, Y., Cai, C., Ling, L., Zeng, L., Wu, M., Wu, Y., Zhang, W., & Liu, Z. (2020). Successful treatment of a centenarian with coronavirus disease 2019 (COVID-19) using convalescent plasma. Transfusion and Apheresis Science. https://doi.org/10.1016/j.transci.2020.102820

Kouakanou, L., Xu, Y., Peters, C., He, J., Wu, Y., Yin, Z., & Kabelitz, D. (2020). VitaminC promotes the proliferation and effector functions of human γδ T cells. *Cellular andMolecular Immunology*, *17* (5), 462–473. https://doi.org/10.1038/s41423-019-0247-8

Kytikova, O. Y., Perelman, J. M., Novgorodtseva, T. P., Denisenko, Y. K., Kolosov, V. P.,Antonyuk, M. V., & Gvozdenko, T. A. (2020). Peroxisome proliferator-activated receptorsas a therapeutic target in asthma. In *PPAR Research* (Vol. 2020). Hindawi Limited. https://doi.org/10.1155/2020/8906968

Le Blanc, K., & Ringdén, O. (2007). Immunomodulation by mesenchymal stem cells and clinical experience. In *Journal of Internal Medicine* (Vol. 262, Issue 5, pp. 509–525). J Intern Med. https://doi.org/10.1111/j.1365-2796.2007.01844.x

Leng, Z., Zhu, R., Hou, W., Feng, Y., Yang, Y., Han, Q., Shan, G., Meng, F., Du, D., Wang, S., Fan, J., Wang, W., Deng, L., Shi, H., Li, H., Hu, Z., Zhang, F., Gao, J., Liu, H., Zhao, R. C. (2020). Transplantation of ACE2- Mesenchymal stem cells improves the outcome of patients with covid-19 pneumonia. Aging and Disease, 11 (2), 216–228. https://doi.org/10.14336/AD.2020.0228

Lin, S. J., Yan, D. C., Lee, W. I., Kuo, M. L., Hsiao, H. S., & Lee, P. Y. (2012). Effect of azithromycin on natural killer cell function. *International Immunopharmacology*, *13* (1), 8– 14. https://doi.org/10.1016/j.intimp.2012.02.013

Liu, Q., Yu, S., Li, A., Xu, H., Han, X., & Wu, K. (2017). Targeting interlukin-6 to relieve immunosuppression in tumor microenvironment. In *Tumor Biology* (Vol. 39, Issue 6). SAGE Publications Ltd. https://doi.org/10.1177/1010428317712445

Lupfer, C., Malik, A., & Kanneganti, T. D. (2015). Inflammasome control of viral infection. In Current Opinion in Virology (Vol. 12, pp. 38–46). Elsevier B.V. https://doi.org/10.1016/j.coviro.2015.02.007

M, H., H, K.-W., N, K., M, M., C, D., & S., P. (n.d.). *The novel coronavirus 2019 (2019- nCoV) uses the SARS-coronavirus receptor 2 ACE2 and the cellular protease TMPRSS2 forentry into target cells.*

Kaplan, J., E. Youd, M., & A. Lodie, T. (2012). Immunomodulatory Activity of Mesenchymal Stem Cells. *Current Stem Cell Research & Therapy*, *6* (4), 297–316. https://doi.org/10.2174/157488811797904353

Magagnoli, J., Narendran, S., Pereira, F., Cummings, T. H., Hardin, J. W., Sutton, S. S., & Ambati, J. (2020). Outcomes of Hydroxychloroquine Usage in United States VeteransHospitalized with COVID-19. *Med*, *January*. https://doi.org/10.1016/j.medj.2020.06.001

Manion, M., Hullsiek, K. H., Wilson, E. M. P., Rhame, F., Kojic, E., Gibson, D., Hammer, J., Patel, P., Brooks, J. T., Baker, J. V., Sereti, I., Carpenter, C., Henry, K., & Overton, E. T. (2017). Vitamin D deficiency is associated with IL-6 levels and monocyte activation in HIV- infected persons. PLoS ONE, 12 (5). https://doi.org/10.1371/journal.pone.0175517

Maoujoud, O., Asserraji, M., Ahid, S., Belarbi, M., & Zemraoui, N. (2020). Anakinra forpatients with COVID-19. In *The Lancet Rheumatology* (Vol. 2, Issue 7, p. e383). LancetPublishing Group. https://doi.org/10.1016/S2665-9913 (20)30177-6

Marik, P. E. (2018). Vitamin C for the treatment of sepsis: The scientific rationale. In Pharmacology and Therapeutics (Vol. 189, pp. 63–70). Elsevier Inc.

https://doi.org/10.1016/j.pharmthera.2018.04.007

Marik, P. E., Khangoora, V., Rivera, R., Hooper, M. H., & Catravas, J. (2017). Hydrocortisone, Vitamin C, and Thiamine for the Treatment of Severe Sepsis and Septic Shock: A Retrospective Before-After Study. *Chest*, *151* (6), 1229–1238. https://doi.org/10.1016/j.chest.2016.11.036

McNamee, E. N., Masterson, J. C., Jedlicka, P., McManus, M., Grenz, A., Collins, C. B., Nold, M. F., Nold-Petry, C., Bufler, P., Dinarello, C. A., & Rivera-Nieves, J. (2011). Interleukin 37 expression protects mice from colitis. *Proceedings of the National Academy of Sciences of the United States of America*, *108* (40), 16711–16716. https://doi.org/10.1073/pnas.1111982108

Mora, J., Schlemmer, A., Wittig, I., Richter, F., Putyrski, M., Frank, A. C., Han, Y., Jung, M., Ernst, A., Weigert, A., & Brüne, B. (2016). Interleukin-38 is released from apoptotic cells to limit inflammatory macrophage responses. Journal of Molecular Cell Biology, 8 (5), 426–438. https://doi.org/10.1093/jmcb/mjw006

Needham, E. J., Chou, S. H. Y., Coles, A. J., & Menon, D. K. (2020). Neurological Implications of COVID-19 Infections. *Neurocritical Care*, *32* (3), 667–671. https://doi.org/10.1007/s12028-020-00978-4

Oh, H. L. J., Gan, S. K. E., Bertoletti, A., & Tan, Y. J. (2012). Understanding the T cell immune response in SARS coronavirus infection. In *Emerging Microbes and Infections* (Vol. 1, Issue 9, p. e23). Taylor & Francis. https://doi.org/10.1038/emi.2012.26

Miguel, P. D., Fuentes-Julian, S., Blazquez-Martinez, A., Y. Pascual, C., A. Aller, M., Arias, J., & Arnalich-Montiel, F. (2012). Immunosuppressive Properties of Mesenchymal Stem Cells: Advances and Applications. *Current Molecular Medicine*, *12* (5), 574–591. https://doi.org/10.2174/156652412800619950

Pandey, S. C., Pande, V., Sati, D., Upreti, S., & Samant, M. (2020). Vaccination strategiesto combat novel corona virus SARS-CoV-2. In *Life Sciences* (Vol. 256, p. 117956). Elsevier Inc. https://doi.org/10.1016/j.lfs.2020.117956

Perez, A., Van Heeckeren, A. M., Nichols, D., Gupta, S., Eastman, J. F., & Davis, P. B. (2008). Peroxisome proliferator-activated receptor-γ in cystic fibrosis lung epithelium. American Journal of Physiology - Lung Cellular and Molecular Physiology, 295 (2). https://doi.org/10.1152/ajplung.90276.2008

Perlman, S., & Dandekar, A. A. (2005). *IMMUNOPATHOGENESIS OF CORONAVIRUS INFECTIONS: IMPLICATIONS FOR SARS*. https://doi.org/10.1038/nri1732

Prompetchara, E., Ketloy, C., & Palaga, T. (2020). Immune responses in COVID-19 and potential vaccines: Lessons learned from SARS and MERS epidemic. In *Asian Pacific Journal of Allergy and Immunology* (Vol. 38, Issue 1, pp. 1–9). Allergy and Immunology Society of Thailand. https://doi.org/10.12932/AP-200220-0772

R, C., & S, P. (n.d.). Pathogenic human coronavirus infections: Causes and consequences of cytokine storm and immunopathology. *Semin Immunopathol*, *39* (5), 529–539.

Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R., & Salem, R. (2020). The COVID-19Cytokine Storm; What We Know So Far. *Frontiers in Immunology*, *11*, 1446. https://doi.org/10.3389/fimmu.2020.01446

Raoult, D., Zumla, A., Locatelli, F., Ippolito, G., & Kroemer, G. (2020). Coronavirus infections: Epidemiological, clinical and immunological features and hypotheses. Cell Stress, 4 (4), 66–75. https://doi.org/10.15698/cst2020.04.216

Rosendaal, F. R. (2020). Review of: "Hydroxychloroquine and azithromycin as a treatment of COVID-19: Results of an open-label non-randomized clinical trial Gautret et al 2010, DOI:10.1016/j.ijantimicag.2020.105949. *International Journal of Antimicrobial Agents*, *56* (1). https://doi.org/10.1016/j.ijantimicag.2020.106063

Rothan, H. A., & Byrareddy, S. N. (2020). The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak. In *Journal of Autoimmunity* (Vol. 109, p. 102433). Academic Press. https://doi.org/10.1016/j.jaut.2020.102433

Saiz, J. C., & Martín-Acebes, M. A. (2017). Reply to Iannetta et al., "Azithromycin shows anti-zika virus activity in human glial cells." *Antimicrobial Agents and Chemotherapy*, *61* (9), 2016–2017. https://doi.org/10.1128/aac.01192-17

Sasazuki, S. (2016). Vitamin C and the common cold. *Japanese Journal of Chest Diseases*,*75* (9), 1002–1005. https://doi.org/10.1079/bjn19920004

Schögler, A., Kopf, B. S., Edwards, M. R., Johnston, S. L., Casaulta, C., Kieninger, E., Jung,A., Moeller, A., Geiser, T., Regamey, N., & Alves, M. P. (2015). Novel antiviral properties of azithromycin in cystic fibrosis airway epithelial cells. *European Respiratory Journal*, *45*(2), 428–439. https://doi.org/10.1183/09031936.00102014

Shakir, L., Javeed, A., Ashraf, M., & Riaz, A. (2011). Metronidazole and the immune system. In *Pharmazie* (Vol. 66, Issue 6, pp. 393–398). https://doi.org/10.1691/ph.2011.0790

Shiratori, H., Feinweber, C., Luckhardt, S., Wallner, N., Geisslinger, G., Weigert, A., & Parnham, M. J. (2018). An in vitro test system for compounds that modulate human inflammatory macrophage polarization. *European Journal of Pharmacology*, *833*, 328–338. https://doi.org/10.1016/j.ejphar.2018.06.017

Smatti, M. K., Al Thani, A. A., & Yassine, H. M. (2018). Viral-induced enhanced disease illness. Frontiers in Microbiology, 9 (DEC), 2991. https://doi.org/10.3389/fmicb.2018.02991

Stegelmeier, A. A., van Vloten, J. P., Mould, R. C., Klafuric, E. M., Minott, J. A., Wootton, S. K., Bridle, B. W., & Karimi, K. (2019). Myeloid cells during viral infections and inflammation. In

Viruses (Vol. 11, Issue 2). MDPI AG. https://doi.org/10.3390/v11020168

Stellari, F. F., Sala, A., Donofrio, G., Ruscitti, F., Caruso, P., Topini, T. M., Francis, K. P., Li, X., Carnini, C., Civelli, M., & Villetti, G. (2014). Azithromycin inhibits nuclear factor- κB activation during lung inflammation: An in vivo imaging study. Pharmacology Research and Perspectives, 2 (5). https://doi.org/10.1002/prp2.58

Stephen-Victor, E., Das, M., Karnam, A., Pitard, B., Gautier, J. F., & Bayry, J. (2020). Potential of regulatory T-cell-based therapies in the management of severe COVID-19. In European Respiratory Journal (Vol. 56, Issue 3). European Respiratory Society. https://doi.org/10.1183/13993003.02182-2020

Sugiyama, K., Shirai, R., Mukae, H., Ishimoto, H., Nagata, T., Sakamoto, N., Ishii, H., Nakayama, S., Yanagihara, K., Mizuta, Y., & Kohno, S. (2007). Differing effects of clarithromycin and azithromycin on cytokine production by murine dendritic cells. *Clinical & Experimental Immunology*, *147* (3), 540–546. https://doi.org/10.1111/j.1365- 2249.2007.03299.x

Sullivan, H. C., & Roback, J. D. (2020). Convalescent Plasma: Therapeutic Hope or Hopeless Strategy in the SARS-CoV-2 Pandemic. In *Transfusion Medicine Reviews*. W.B. Saunders. https://doi.org/10.1016/j.tmrv.2020.04.001

Sun, X., Hou, T., Cheung, E., Iu, T. N. T., Tam, V. W. H., Chu, I. M. T., Tsang, M. S. M., Chan, P. K. S., Lam, C. W. K., & Wong, C. K. (2020). Anti-inflammatory mechanisms of the novel cytokine interleukin-38 in allergic asthma. *Cellular and Molecular Immunology*, *17* (6), 631–646. https://doi.org/10.1038/s41423-019-0300-7

Tan, L., Wang, Q., Zhang, D., Ding, J., Huang, Q., Tang, Y.-Q., Wang, Q., & Miao, H. (2020). Lymphopenia predicts disease severity of COVID-19: A descriptive and predictive study. *Signal Transduction and Targeted Therapy*, *5* (1), 33. https://doi.org/10.1038/s41392-020-0148-4

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). Il-6 in inflammation, Immunity, and disease. *Cold Spring Harbor Perspectives in Biology*, *6* (10). https://doi.org/10.1101/cshperspect.a016295

Tang, C., Wang, Y., Lv, H., Guan, Z., & Gu, J. (2020). Caution against corticosteroid-basedCOVID-19 treatment. In *The Lancet* (Vol. 395, Issue 10239, pp. 1759–1760). Lancet Publishing Group. https://doi.org/10.1016/S0140-6736 (20)30749-2

Targher, G., Pichiri, I., & Lippi, G. (2012). Vitamin D, thrombosis, and hemostasis: More than skin deep. *Seminars in Thrombosis and Hemostasis*, *38* (1), 114–124. https://doi.org/10.1055/s-0031-1300957

Tay, M. Z., Poh, C. M., Rénia, L., MacAry, P. A., & Ng, L. F. P. (2020). The trinity of COVID-19: Immunity, inflammation and intervention. In *Nature Reviews Immunology* (Vol. 20, Issue 6, pp. 363–374). Nature Research. https://doi.org/10.1038/s41577-020-0311-8

Thorlund, K., Dron, L., Park, J., Hsu, G., Forrest, J. I., & Mills, E. J. (2020). A real-time dashboard of clinical trials for COVID-19. In *The Lancet Digital Health* (Vol. 2, Issue 6, pp. e286–e287). Elsevier Ltd. https://doi.org/10.1016/S2589-7500 (20)30086-8

Tilg, H., Dinarello, C. A., & Mier, J. W. (1997). IL-6 and APPs: Anti-inflammatory and immunosuppressive mediators. In *Immunology Today* (Vol. 18, Issue 9, pp. 428–432). Elsevier Ltd. https://doi.org/10.1016/S0167-5699 (97)01103-1

TW, L., TK, K., & D, T. (n.d.). Acute respiratory distress syndrome in critically ill patientswith severe acute respiratory syndrome. *JAMA*, *290* (3), 374–380.

Ucciferri, C., Auricchio, A., Di Nicola, M., Potere, N., Abbate, A., Cipollone, F., Vecchiet,J., & Falasca, K. (2020). Canakinumab in a subgroup of patients with COVID-19. In *The Lancet Rheumatology* (Vol. 2, Issue 8, pp. e457-ee458). Lancet Publishing Group. https://doi.org/10.1016/S2665-9913 (20)30167-3

Ulrich, H., & Pillat, M. M. (2020). CD147 as a Target for COVID-19 Treatment: SuggestedEffects of Azithromycin and Stem Cell Engagement. *Stem Cell Reviews and Reports*, *16* (3), 434–440. https://doi.org/10.1007/s12015-020-09976-7

Velazquez-Salinas, L., Verdugo-Rodriguez, A., Rodriguez, L. L., & Borca, M. V. (2019). The Role of Interleukin 6 During Viral Infections. *Frontiers in Microbiology*, *10* (MAY), 1057. https://doi.org/10.3389/fmicb.2019.01057

Veronese, N., Demurtas, J., Yang, L., Tonelli, R., Barbagallo, M., Lopalco, P., Lagolio, E., Celotto, S., Pizzol, D., Zou, L., Tully, M. A., Ilie, P. C., Trott, M., López-Sánchez, G. F., &Smith, L. (2020). Use of Corticosteroids in Coronavirus Disease 2019 Pneumonia: A Systematic Review of the Literature. *Frontiers in Medicine*, *7*, 170. https://doi.org/10.3389/fmed.2020.00170

Vincent, M. J., Bergeron, E., Benjannet, S., Erickson, B. R., Rollin, P. E., Ksiazek, T. G.,Seidah, N. G., & Nichol, S. T. (2005). Chloroquine is a potent inhibitor of SARS coronavirusinfection and spread. *Virology Journal*, *2*, 1–10. https://doi.org/10.1186/1743-422X-2-69

Wan, S., Yi, Q., Fan, S., Lv, J., Zhang, X., Guo, L., Lang, C., Xiao, Q., Xiao, K., Yi, Z., Qiang, M., Xiang, J., Zhang, B., & Chen, Y. (2020). Characteristics of lymphocyte subsets and cytokines in peripheral blood of 123 hospitalized patients with 2019 novel coronavirus Pneumonia (NCP). MedRxiv, 2020.02.10.20021832. https://doi.org/10.1101/2020.02.10.20021832

Wang, L., Quan, Y., Yue, Y., Heng, X., & Che, F. (2018). Interleukin-37: A crucial cytokinewith multiple roles in disease and potentially clinical therapy (Review). In *Oncology Letters*(Vol. 15, Issue 4, pp. 4711–4719). Spandidos Publications. https://doi.org/10.3892/ol.2018.7982

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., Fu, S., Gao, L., Cheng, Z., Lu, Q., Hu, Y., Luo, G., Wang, K., Lu, Y., Li, H., Wang, S., Ruan, S., Yang, C., Mei, C., … Wang, C. (2020). Remdesivir in adults with severe COVID-19: A randomised, double-blind, placebo-controlled,

multicentre trial. *The Lancet, 395* (10236), 1569–1578. https://doi.org/10.1016/S0140-6736 (20)31022-9

WHO. (2020). Coronavirus disease (COVID-19) situation report—119. Williams, P. C. M., & Berkley, J. A. (2018). Guidelines for the treatment of dysentery (shigellosis): A systematic review of the evidence. Paediatrics and International Child Health, 38, S50–S65. https://doi.org/10.1080/20469047.2017.1409454
Wong, C. K., Lam, C. W. K., Wu, A. K. L., Ip, W. K., Lee, N. L. S., Chan, I. H. S., Lit, L.C. W., Hui, D. S. C., Chan, M. H. M., Chung, S. S. C., & Sung, J. J. Y. (2004). Plasma inflammatory cytokines and chemokines in severe acute respiratory syndrome. *Clinical and Experimental Immunology*, *136* (1), 95–103. https://doi.org/10.1111/j.1365- 2249.2004.02415.x

Xie, Y., Li, Y., Cai, X., Wang, X., & Li, J. (2016). Interleukin-37 suppresses ICAM-1 expression in parallel with NF-κB down-regulation following TLR2 activation of human coronary artery endothelial cells. *International Immunopharmacology*, *38*, 26–30. https://doi.org/10.1016/j.intimp.2016.05.003

Xu, Z., Shi, L., Wang, Y., Zhang, J., Huang, L., Zhang, C., Liu, S., Zhao, P., Liu, H., Zhu, L., Tai, Y., Bai, C., Gao, T., Song, J., Xia, P., Dong, J., Zhao, J., & Wang, F. S. (2020). Pathological findings of COVID-19 associated with acute respiratory distress syndrome. *TheLancet Respiratory Medicine*, *8* (4), 420–422. https://doi.org/10.1016/S2213-2600 (20)30076-X

Yan, X., Xie, B., Wu, G., Hu, J., Wang, D., Cai, X., & Li, J. (2019). Interleukin-37: The effect of anti-inflammatory response in human coronary artery endothelial cells. In *Mediators of Inflammation* (Vol. 2019). Hindawi Limited. ps://doi.org/10.1155/2019/2650590

Yang, J., Yang, X., Yang, J., & Li, M. (2018). Hydroxychloroquine inhibits the differentiation of Th17 cells in systemic lupus erythematosus. *Journal of Rheumatology*, *45* (6), 818–826. https://doi.org/10.3899/jrheum.170737

Yang, Y., Xiao, Z., Ye, K., He, X., Sun, B., Qin, Z., Yu, J., Yao, J., Wu, Q., Bao, Z., & Zhao, W. (2020). SARS-CoV-2: Characteristics and current advances in research. In *VirologyJournal* (Vol. 17, Issue 1). BioMed Central. https://doi.org/10.1186/s12985-020-01369-z

Yi, T., & Song, S. U. (2012). Immunomodulatory properties of mesenchymal stem cells andtheir therapeutic applications. In *Archives of Pharmacal Research* (Vol. 35, Issue 2, pp.213–221). Arch Pharm Res. https://doi.org/10.1007/s12272-012-0202-z

Yuen, K. S., Ye, Z. W., Fung, S. Y., Chan, C. P., & Jin, D. Y. (2020). SARS-CoV-2 and COVID-19: The most important research questions. *Cell and Bioscience*, *10* (1). https://doi.org/10.1186/s13578-020-00404-4

Zhang, J., Xie, B., & Hashimoto, K. (2020). Current status of potential therapeutic candidates for the COVID-19 crisis. In *Brain, Behavior, and Immunity* (Vol. 87, pp. 59–73). Academic Press Inc. https://doi.org/10.1016/j.bbi.2020.04.046

Zhang, W., Zhao, Y., Zhang, F., Wang, Q., Li, T., Liu, Z., Wang, J., Qin, Y., Zhang, X., Yan, X.,

Zeng, X., & Zhang, S. (2020). The use of anti-inflammatory drugs in the treatmentof people with severe coronavirus disease 2019 (COVID-19): The experience of clinical immunologists from China. In *Clinical Immunology* (Vol. 214, p. 108393). Academic Press Inc. https://doi.org/10.1016/j.clim.2020.108393

Zhang, Y., Wang, X., Li, X., Xi, D., Mao, R., Wu, X., Cheng, S., Sun, X., Yi, C., Ling, Z., Ma, L., Ning, Q., Fang, Y., Sun, B., & Wu, D. (2020). Potential contribution of increased soluble IL-2R to lymphopenia in COVID-19 patients. In *Cellular and Molecular Immunology* (Vol. 17, Issue 8, pp. 878–880). Springer Nature.https://doi.org/10.1038/s41423-020-0484-x

Review Article

Vitamin E, a potent antioxidant and modulator of Alzheimer's disease pathologies

Mahima Raval[a], Anand Krishna Tiwari[a]*

[a] *Department of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institutional Area, Gandhinagar 382426, India*

Email: anandk.tiwari@iar.ac.in

Abstract

Human body is continuously exposed to a variety of substances, compounds, or several intracellular processes that produce reactive oxygen species that may be very dangerous to several cellular processes, adversely affecting the intracellular defence system such as antioxidant enzyme level and ultimately human health. According to a number of studies, adding antioxidants to the diet strengthens the body's defence mechanism and intracellular antioxidant enzyme levels. Plants and plants-based compounds are the rich source of several antioxidant compounds and since civilization humans are dependent on plants and plant-based compounds for nutrition and several health-related issues. There are a variety of antioxidants that can be helpful to improve the well-being of humans in different disease conditions. In recent years, neurodegenerative diseases such as AD have become a major challenging problem across the globe and need immediate concern due to aberrant increase in the numbers. Several researches are trying to find the therapeutic targets to treat these diseases but still no fruitful drug is discovered. Several studies have suggested that dietary supplementation of antioxidants improve the health span of AD patients. This review is focused on the use of Vitamin E, an antioxidant and its possible therapeutic potential to treat/prolong/modulate the AD pathologies in humans.

Keywords: vitamin E, tocopherol, anti-oxidant, reactive oxygen species (ROS), AD, amyloid B42

Introduction

Antioxidant compounds are plant derived, natural or synthetic molecules that scavenge free radicals and associate cellular damage (Szymanska et al., 2016; Cui et al., 2020; Michalak 2022; Szymanska et al., 2018; Stoia and Oancea, 2022; Augustyniak et al., 2010; Uzombah, 2022). Antioxidants may be in enzymatic or non-enzymatic forms intra/extracellularly (Nimse and Pal, 2015; Irato and Santovito, 2021; Moussa et al., 2019; Kumar et al., 2014). There are various factors (pollutants, stress, etc) that have potential to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lodovici and Bigagli, 2011; Lelieveld et al., 2021; Mazuryk et al., 2020; Lakey et al., 2016;

Metodiewa and Kośka, 1999). These ROSand RNS may alter the cellular homeostasis resulting in altered cellular physiology (Di Meoet al., 2016; Zarkovic, 2020; He et al., 2017; Dunn et al., 2015) and ultimately end up in disease conditions (Afanas, 2011; Liu et al., 2018; Mijatović et al., 2020; Ahmad et al., 2017). In order to cope up with ROS/RNS conditions, cell has evolved with intracellular defence systems in the form of antioxidant genes encoding antioxidant enzymes such as *superoxide dismutase* (SOD), *catalase* (CAT), *glutathione peroxidase* (GSHPx), etc (Bandoet al., 2005; Aguilar et al., 2016; Eddaikra and Eddaikra, 2021; Ighodaro and Akinloye, 2018). Under stress conditions, the level of these antioxidant enzymes is increased to fight against the oxidative stress (Blokhina et al., 2003; Sharifi-Rad et al., 2020).

This review is focused on potential of natural antioxidant Vitamin E and its possible application as a therapeutic agent in the treatment of Alzheimer's disease (AD). Vitamin E is a fat-soluble vitamin with great antioxidant property and acts as a chain breaker in lipid peroxidation (Stevens et al., 2021; Bellows et al., 2012; Johnson and Mohn, 2015). Its molecular formula is $C_{29}H_{50}O_2$ and is a natural antioxidant that possesses lipophilic radical scavenging activity (Niki, 2015; Niki and Abe, 2019). Several lines of evidence has suggestedthat the use of Vitamin E is beneficial in the treatment/cure of the cognitive impairment and AD (Farina et al., 2017; Lloret et al., 2019; Kryscio et al., 2017; Pavlik et al., 2009; Boothby and Doering, 2005; Isaac et al., 2008; Birks, 2000).

Vitamins are organic molecules that are important micronutrients needed by an organism for the proper functioning of the cellular processes (Thomas, 2006). Vitamins are essential nutrients, which mean they are not synthesised by the body but are needed by the body for its physiological functioning, with one exception that some organisms and not all can synthesise vitamin C (Tardy et al., 2020; Godswill et al., 2020; Drouin et al., 2011; Padayatty and Levine, 2016).

Vitamin E, as mentioned above is a fat-soluble compound and was first discovered by Evans Bishop in 1922 (Evans and Bishop, 1922; Bell, 1987; Rizvi et al., 2014). Of all known vitamins, the term vitamin E is used to describe a class of eight lipophilic molecules with a chromanol ring and a saturated or unsaturated carbon phytyl side chain (Wang, X., and Quinn et al., 1999; Niki and Abe, 2019; Reboul, 2017). These are categorised into two groups including tocopherols with a chromanol ring and the saturated side chain including α, β, γ, δtocopherols and tocotrienols with unsaturated side chain with three double bonds including α, β, γ, δ tocotrienols (Ahsan et al., 2015; Szewczyk et al., 2021; Aggarwal et al., 2010; Singh et al, 2013; Yaseen et al., 2019; Sen et al., 2006; Fu et al., 2014). Tocopherols follow the RRR configuration at position 2', 4', and 8' positions whereas tocotrienol follows R configuration only at 2' position (Kuchan et al., 2018; Atanasiu et al., 2006; Ranard et al., 2018; Jiang, 2022). The isoforms i.e. α, β, γ, δ tocopherols and tocotrienols are differentiated on the basis of number of H or methyl group and its localisation as for instance 5' or 7' position of the chromanol ring (Aggarwal et al., 2010; Szewczyk et al., 2021; Wallert et al.,2020). Both tocopherols

and tocotrienols are evident to be the potent antioxidants because of its ability to skulk the peroxyl radicals, breaking the propagating chain (Azzi, 2019; Shahidi et al., 2016; Munné-Bosch and Alegre, 2002; Edwards et al., 2022).

It has been reported that all the isoforms of tocopherols and tocotrienols show similar antioxidant ability because of the similar phenolic moieties they possess, although the synthetic form α-tocopherol is preferred by the body (Burton and Ingold, 1981; Jiang et al., 2001). When compared with other isoforms, the α-tocopherol potency is approximately the same when measured in organic solution, or solutions mimicking biological membranes (Mukai et al., 1989; Pryor et al., 1993; Pryor et al., 1988; Mukai et al., 2007; Leth et al., 1977), but the most preferred one is α-tocopherol due to higher metabolism of non-α- tocopherol forms and favouring of α-tocopherol by hepatic systems. As a free radical scavenger, Vitamin E skulks the hydroxyl radicals by breaking the propagating chain where the phenol group on the chromanol ring donates a hydrogen atom to the free radical, generating a hydroperoxide and a tocopheroxyl radical which is enough stable to inhibit the propagating chain which then interacts with another radical giving inactive products or in presence of vitamin C can re-establish it to their indigenous form (Theriault et al., 1999; Constantinescu et al,1993; Vatassery et al., 1995; Thiele et al.,2001).

All these isoforms are basically synthesised by plants and are exhibited to different extents in fat rich foods for example, edible oils including oils from soybean, canola, sunflower seeds, nuts, and rarely in fruits and vegetables. α-tocopherol is found in peanuts and almonds, while γ- tocopherol is obtained from walnuts, sesame seeds, etc (McLaughlin and Weihrauch, 1979; Chun et al., 2006; Dreher and ML, 2012). In addition to its antioxidant activity, the dietary uptake of this group of essential micronutrients is important because they are involved in maintaining the integrity of the cell membranes and are also involved in the development of tissues and organs including brain, and are employed in signalling pathways (Galli et al., 2017; Wang and Quinn, 2000; Howard et al., 2011; Conti et al., 1990; Siddiqui et al., 2021).

As mentioned above, α-tocopherol is the most abundant isoform of vitamin E present in the body and is nicely absorbed by the hepatic system. Various studies have demonstrated that this absorption of the α-tocopherol is done by a specialised protein molecule known as α - tocopherol transfer protein (α-TTP) associated with an (ABCA1) that aids in the integrationof α-tocopherols to the lipoproteins from where this is transported throughout the body in different tissues (Manor and Morley, 2007; Stocker and Azzi, 2000; Ulatowski et al., 2022; Arita et al., 1997). The higher affinity of the α-TTP for alpha-tocopherol makes the difference preventing its metabolization, whereas other isoforms are not bound to this transfer protein being available for its own ω-hydroxylase, which functions in the hydroxylation and further processing of these isoforms generating metabolites with different length side chains such as 13'-hydroxychromanol, carboxy chromanols, including 3'-carboxychromanol and other terminal metabolites. This difference among the interactions leads to the accumulation of the

unmetabolized α-tocopherol and the metabolite resultants of the other isoforms including γ-T and tocotrienols (Brigelius Flohé, R., and Traber, 1999; Jiang et al., 2001; Manor and Morley, 2007). However, other conflicting studies have suggested that tocotrienols are better scavenging molecules than tocopherols because of greater levels of its distribution throughout the phospholipid bilayer and due to its property to effectively counteract the lipid peroxyl radicals (Packer et al., 2001; Wong et al., 2012). (Niki, 2007) reviewed that tocopheryl hydroquinone, the reduced form of α-TQ is a potent radical scavenging antioxidant oxidant in-vivo, in which reaction α-T is oxidised to α-TQ, suggesting α-T as a promising anti- oxidant isoform in treatment of AD.

(Bahadorani et al., 2008) in their study took three vitamins i.e. retinol, ascorbic acid, and α – tocopherol to check its effects on *Drosophila* life span under normal and oxidative stress conditions. The results of α -tocopherol in both conditions is a matter of concern. Studying its effects under normal conditions found no visible and significant effect on longevity. When wild type flies were fed vitamin E under stress conditions, it was observed that both the average and maximum life span increased. The 25 IU/mL dose of vitamin E reflected the largest life span ((~8 days) when compared to the control and the other two vitamins.

It is a well-known fact that organisms in their lifetime are exposed to certain compoundsand conditions which are harmful and cause generation of oxidative stress affecting human health and needs to be excavated. Thus, to ascertain the function of vitamin E as a dietary supplement, researchers (Bahadorani et al., 2008) used Cu/Zn SOD1 deficient flies, a cytoplasmic antioxidant acting as a superoxide radical scavenger. They discovered that in this condition, out of the various concentrations fed, 25I U/ml showed increase in the life span of these deficient flies (up to 32 days), 15 days more when compared to control.They even determined the total antioxidant capacity of both wild type and SOD1 deficient flies using Trolox antioxidant assay and illustrated that α-tocopherol significantly increases the total antioxidant capacity of SOD1 deficient flies and a minor increase of this total antioxidant capacity in the wild type flies also.

Neurodegenerative diseases including AD are suggested to be multifactorial which means that this is not an outcome of any one altered condition but has many factors contributing towards it, including defective protein degradation and aggregation, oxidative stress generation, impaired mitochondrial function, exposure to metal toxicity, age, lifestyle, genetics, etc (Sheikh et. al. 2013; Ibrahim and Gabr, 2019; Samanta and Govindaraju, 2022; Jellinger, 2010; Martier and Konstantinova, 2020). Among all these factors, age and age related excessive oxidative stress is a matter of major concern and is reported to be the most important factor for the onset of Alzheimer' s disease (Koedam et al., 2010; Reitz et al., 2020; Cassidyet al., 2020; Nunomura et al., 2006; Chen and Zhong, 2014).

The free radical theory was first postulated by Harman in 1956 (Han et al., 2014). Free radicals are atoms or molecules that have an unpaired electron (single) in its outermost orbit,making it very unstable (Phaniendra et al., 2015; Di Meo et al., 2020; Webster et al., 1988; Martemucci et al., 2022).

As electrons prefer to be in pairs and free radicals are created when a single electron is in their outer orbit, these radicals seek for more electrons from nearby molecules, such as DNA, proteins, and lipids in the cells, harming the structure and associated processes. With relation to NDs, the excessive production of free radicals, also known as oxidative stress, results in the loss of neurons (Tuppo et al., 2001; Phaniendra et al., 2015; Petersen et al., 2017; Sharifi-Rad et al., 2020; Khanna et al., 2014). This production of free radicals is indeed needed for the basic molecular processes including intracellular signalling but when in excess, it leads to oxidative damage (Lobo et al., 2010; Dröge, 2002; Valko et al., 2007; Tvrdá et al., 2017; Khan et al., 2018). The free radicals are categorized into two types; Reactive Nitrogen Species (RNS) and Reactive Oxygen Species (ROS) (Ozcan and Ogun, 2005; Phaniendra et al., 2015; Radi, 2008; Shields et al., 2021). The ROS generated are the reduction products of oxygen to H_2O in mitochondria. ROS, for instance, superoxide ion, H_2O_2 (hydrogen peroxide), hydroxyl radical, and water are primary radicals produced in aerobic processes, electron leak from electron transport chain (ETC) complexes, and enzymatic reactions using oxygen; while endogenous antioxidant systems eliminate these radicals (Fukai and Ushio-Fukai, 2011; Lloret et al., 2019; Birch- Machin, M. A., and Turnbull, 2001; Zorov et al., 2014; Johannsen and Ravussin, 2009). Along with the endogenous generators of free radical, several other oxidants play an important role in generation including, virus and bacteria infected cells generating free radicals in the process of phagocytosis, by-products of cytochrome p450, and the environmental factors such as UV light and cigarette smoke (Akaike, 2001; Veith and Moorthy, 2018; Namazi, 2009; Rice-Evans et al., 1995). As mentioned above, the lipids can also be oxidized. Free radicals when generated can cause lipid peroxidation, leading to disintegration of the cell membranes, rupturing of the organelles, and ultimately cell death (Ayala et al., 2014; Repetto et al., 2012; McCay and Poyer et al., 1976; Praticò, 2002). Moreover, it has been observed that these free radicals can oxidise proteins and nucleic acids, which can result in the production of reactive carbonyls and protein nitrates (Semchyshyn et al., 2014; Nunomura et al., 1999; Altomare et al., 2021; Fritz et al., 2013).

It has been widely reported that as people age, there is an increase in the production of free radicals and a decrease in the antioxidant enzymes that act as scavengers, which leads to the development of oxidative stress, which is a key indicator of AD (Tönnies and Trushina, 2017; Huang et al., 2016; Misrani et al., 2021; Sharma and Kim, 2021; Simpson and Oliver, 2020). This condition arises in the brain frequently, as it consumes 25-30% of total oxygen. With a dry weight of 2-3% of body, the tendency of the brain to consume this much amount of oxygen makes it a susceptible organ for this condition to arise (Vadas et al., 2017; Rink and Khanna, 2011). According to (Behl, 1999), AD may develop as a result of some brain regions being exposed to free radicals and as a result of the endogenous defence systems functioning less effectively. Another study revealed an increase in the oxidation of one of the PUFAs, arachidonic acid, in the brain, which produced stable products called F2 isoprostanes, which are indicators of in vivo oxidative stress in the brains of AD patients (Pratic

et al., 1999).

AD is reported to be the most prevalent NDDs, that affects 10% of the population over the age of 65 and 50% of the population over the age of 85 (Zhang et al., 2011). AD is named after German psychiatrist and neuropathologist, Dr Alois Alzheimer's in 1906 (Hippius and Neundörfer, 2022; DeTure and Dickson, 2019; Small and Cappai, 2006; Tagarelli et al., 2006). The disease is reported to affect different brain regions including cerebral cortex, basal ganglia, thalamus, and hippocampus associated with executive functions, reward, motor, sensory functions, and importantly memory starting from early stages of AD (Gan et al., 2018; Raji et al., 2009; Planche et al., 2022; Hamasaki et al., 2019; Rao et al., 2022). The disease is characterized by the presence of two pathological hallmarks including senile plaques of insoluble amyloid beta (Aβ) peptides of 42 amino acids that gets accumulated extracellularly between the neurons and another is the presence of neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein intracellularly (Tillement and Papadopoulos, 2011; Serrano-Pozo et al., 2011; Skender-Gazibara and Slobodan, 2001; Liebscher and Meyer-Luehmann, 2012). The disease follows certain stages which will bring about changes in the person who is affected and the stages include early, middle, and late. As the disease affects differently, the symptoms, the order in which they appear, and the duration, varies from person to person (Dassa and Amir, 2014; Muck-Seler et al., 2009; Haak, 2003; Adams and Sanders, 2004; Hopper et al., 2001). The symptoms of early-stage AD includes forgetfulness, communication difficulties, anomia, executive function, and changes in mood and behavior. The symptoms of middle stage include patients having difficulty in performing daily chores. They especially face issue with maintaining personal hygiene. The patients in the late stage AD experience the worst symptoms including temporary or permanent memory loss, urinary or stool incontinence, and severe disorientation (Frozza et al., 2018; Jahn, 2022; Kirova et al., 2015).

The formation of senile plaques is an outcome of abnormal cleavage of amyloid precursor protein (APP) (O'brien and Wong, 2011; Dang et al., 2015; Xu et al., 2016; Sun et al., 2015). APP is an integral transmembrane protein that is reported to be expressed in many different types of tissues but is concentrated in the synapse of neurons (Chen et al., 2017; Selkoe et al.,1988; Galloway et al., 2007; Herzog et al., 2004; Lee et al., 2008; Akaaboune et al., 2001). APP is a 695 amino acid protein that has a large ectodomain and a comparatively smaller intracellular region (Kang et al., 1987; Dyrks et al., 1988; Siman, 1994; Singh et al., 2008). Under normal conditions, this APP gets cleaved in the non-amyloidogenic pathway where first the APP is cleaved by an endoprotease α-secretase into secreted APP α (s-APPα) and C-Terminal Fragment α (CTFα) of 83 amino. This CTFα then successively is cleaved by another endoprotease known as γ-secretase into a P3 fragment and APP intracellular domain (AICD) (Lichtenthaler, 2012; Zhang et al., 2011; Adeniji et al., 2017; Sontheimer, 2015; Chow et al., 2010; Zhang and Song, 2013). The APP cleaved under normal

conditions has beenhypothesized to play a role in synapse formation and Blood Brain Barrier (BBB) protection (Hoe et al., 2012; Montagna et al., 2017; Caldwell et al., 2013; Ristori et al., 2020). However, APPcleaved in the amyloidogenic pathway is reported to cause the production of insoluble Aβ peptides. Under abnormal conditions that might include increasing age, individual health status, and even genetic mutations in the γ-secretase genes including *PSENI* and *PSENII*, theamyloidogenic pathway is followed (Kelleher III and Shen, 2017; Thinakaran, 1999), where initially the APP is cleaved by endoprotease β-secretase into secreted APP β (s-APPβ) and CTFβ of 83 amino acids. This CTFβ is then abnormally cleaved by γ-secretase into insolubleAβ peptides of 40-42 amino acids and AICD. This generation of insoluble Aβ peptides are reported to get aggregated extracellularly into senile plaques (Murpy and LeVine III, 2010; Pluta et al., 2013; Chow et al., 2010; Zhang and Song, 2013; Korabecny et al., 2018) and these are widely reported to play a crucial role in the generation of excessive ROS lead Oxidativestress (OS), mitochondrial dysfunction including malfunctioning ETC, ATP synthesis; however, it may also have antimicrobial activity (Gosztyla et al., 2018; Maes et al., 2011; Shan, 2005; Yuste et al., 2015; Chen, and Yan, 2007; Wang et al., 2008; Cha et al., 2012).The other hallmark of AD includes the formation of NFTs of hyperphosphorylated tau protein (Nelson et al., 2009; Kazim and Iqbal, 2016; Armstrong, 2006; DeTure and Dickson, 2019; Skender-Gazibara and Slobodan, 2001), that is microtubule associated protein and plays an important role in assembly of the microtubule track and its maintenance (Mietelska- Porowska et al., 2014; Barbier et al., 2019; Kadavath et al., 2015). The maintenance function of tau is played by homeostatic phosphorylation and dephosphorylation of this protein (Benítez et al., 2021; Barbier et al., 2019; Kolarova et al., 2012); however, hyperphosphorylation of this protein causes dyshomeostasis leading to the aggregation of hyperphosphorylated tau protein into tangles and loss of microtubule track intracellularly in the axon of neuron (O'Neill et al., 2001; Takashima et al., 2016; Jie et al., 2021; Gong and Iqbal, 2008). This intracellular accumulation of NFTs is reported to cause a number of anomalies including blockage of the axons that cause impaired transfer of mitochondria to axon terminals leading to impaired synapse formation as these mitochondria travelling acrossthe axons are employed to provide ATP for the synapse formation and mitochondrial dysfunction. It is also reported to cause impaired mitochondrial dynamics including fission and fusion and also increases the mitochondrial elimination through mitophagy (Adalbert et al., 2018; Salvadores et al., 2020; Kopeikina et al., 2011; Eckert et al., 2011; Correia et al., 2016; Cai and Tammineni, 2016).

As oxidative stress is a prime marker for AD the important cause of it is thought to be peroxidation of lipids, amyloid beta aggregation, exposure to toxic metals etc (Huang et al.,2016; Gella and Durany 2009; Markesbery 1999; Tamagno et al., 2021; Butterfield et al., 2013; Birla et al., 2020). It has been proposed that extracellular Amyloid beta peptide aggregation in AD is a source of free radical generation that causes neurotoxicity in synaptosomal membranes in AD patients. According to another study, advanced glycation end products, which are post-translationally modified proteins

created when reduced sugars react with protein side chains and undergo further modification in a three-step process carried out by iron, are present and act as potent sources of free radical generating substances. These AGEs were also reported to be present in the senile plaques of amyloid beta peptide in case of AD (Munch et al., 1998; Castellani et al., 2001; Lloret et al., 2019). Studies by (Lovell et al., 1995; Arlt et al., 2002) have shown that the level of thiobarbituric acid reactive substances and 4- hydroxy-2-nonenal, a measure of lipid peroxidation, was increased in brains of patients with AD. (Cassarino et al., 1999; Berman and Brodaty, 2004) has explained that in the course of increasing age, the mitochondrial electron transport chain becomes less competent which might play a role in free radical generation, ultimately increasing oxidative stress.

Iron has been linked to neurofibrillary tangles and beta amyloid deposits in the brain; this connection of iron with iron has also been noted above. The body's imbalance of metal ions, such as iron and copper, has been shown to be the primary source of free radical generation (Rottkamp et al., 2000; Berman and Brodaty, 2004). In addition to all of these issues, beta amyloid accumulation in the brain can significantly contribute to inflammation, which in turn increases oxidative stress (Pohl et al., 2019).

Additionally, whenever superoxide radicals are produced, they react with nitric oxide, which primarily has anti-inflammatory and anticoagulant properties, inducing the inactivation of those properties and producing peroxynitrite, a major oxidant that increases the susceptibility of neuronal cells to oxidative stress (Uttara et al., 2009; Gandhi et al., 2012; Guzik, 2006; Fukai et al., 2011). Considering the excessive oxidative damage in the case of neurodegeneration, there is no doubt that researchers are using a wide range of antioxidants in order to prevent or delay the course of the diseases associated with neuronal loss.

Endogenous antioxidant defence mechanism is already employed by nature in order to prevent the oxidative damage caused in the course of aging and other physiological activities.Studies by various researchers have demonstrated that in the course of aging, the imbalancebetween the increased oxidative stress and the endogenous mechanism leads to decrease in the efficient activity of the endogenous antioxidants leading to oxidative damage conditions.The endogenous antioxidants can be categorised into two groups, enzymatic and non- enzymatic. The enzymatic endogenous antioxidants include, superoxide dismutases, catalase, glutathione peroxidase, and glutathione reductase, while the non-enzymatic includes ascorbic acid, polyphenols, lipoic acid, NADPH (Augustyniak et al., 2010; Pohl et al., 2019). The incidence of oxidative stress is first encountered by a class of endogenous antioxidants, the SOD class having three different isoforms and localised in different subcellular positions including the SOD1 (Cu/Zn SOD) in the cytoplasm, mitochondria; SOD2 (Mn SOD) in the mitochondrial matrix; and the extracellular SOD with the appropriate metal cofactors having (catalytic and stability functions). SODs are responsible for the dismutation of superoxide radicals to hydrogen peroxide, wherein this process further catalysis to H_2O which is done by

catalase, glutathione peroxidases. In addition to this function, SODs are responsible for inhibiting the inactivation of nitric oxide which is involved in signalling, anticoagulant and anti-inflammatory functions,and also formation of peroxynitrite by superoxide radicals (Fukai et al., 2011; Abreu et al., 2010; Guzik et al., 2006; Madamanchi et al., 2007). However, evidence has suggested that the endogenous defence systems become less abundant with age and the repair mechanism gets impaired as the cells grow old, increasing the need for exogenous dietary antioxidant uptake by the individual.

Role of Vitamin E related to AD

As mentioned above, Vitamin E is made up of fat soluble compounds and plays several different roles in the treatment of AD. Vitamin E majorly plays a role of an antioxidant but it also plays some unrelated roles such as a regulator of signal transduction, gene expression, redox sensor, modulator of specific cell function via interaction with certain membrane domain.

(Kakkar et al., 1996) have demonstrated that Vitamin E deficiency in experimental animals induces pathological changes in the reproductive, cardiovascular, and nervous systems and in other tissues. In addition, they have demonstrated that vitamin E supplementation LT50 and LT100 life spans of *Z. paravittiger* supporting the results of previous studies on rotifersand nematodes, predicted the increase in life spans as a function of vitamin E as a free radical scavenger. They concluded that a lower concentration feeding of vitamin E (1,5, 10 microgram/ml) but not higher concentration is responsible for the above results. They also measured the levels of malondialdehyde, a carbonyl compound produced during the process of lipid peroxidation, in both sexes to determine how many free radicals were produced. They found that there was a significant decrease in the MDA content at all intervals in the 43 days old lifespan of the flies fed on optimum concentration 5 micro gm/ml of vitamin E in comparison to control (Day 1 male- 7.74 +-0.37 in control and decreased to 5.77+-0.21 in vitamin E fed flies, wherein in females the concentration was lower as compared to control and the vitamin E fed males at 6.45 +-0.18 in control and 5.10 +-0.28 in vitamin E fed flies).

(Gohil et al., 2003) have shown that low α-tocopherol levels induce down regulation of genes involved in myelination and synaptogenesis, neuronal vesicle transport, and glial functions.(Li et al., 1999) have shown that γ-tocopherol and tocotrienols showed systematic *superoxidedismutase* activity as compared to α -tocopherol in AD. Another study showed that, in NDs,Aβ protein, a pathological hallmark of AD induces toxicity through an oxidative stress mechanism wherein high hydrogen peroxide production leads to nerve cell death and finally AD. Vitamin E as suggested, blocks the formation of hydrogen peroxide by inhibiting free radical chain reaction.

(Sung et al., 2004) suggested that supplementing vitamin E before the appearance of pathology of AD, supresses brain lipid peroxidation, mitigates brain oxidative stress, and significantly reduces the

Aβ forming levels and senile plaque deposition in Tg2576 young mice model but not in the aged. In their approach to determine whether vitamin E supplementation reduces the formation of the amyloid peptides, they took (Tg2576) transgenic mice model, wherein they checked for the levels of Aβ 1-40 and Aβ 1-42 in high salt and formic acid soluble fractions of brain homogenates. They found that 13 months old mice showed an increase in the levels of both peptide forms in the hippocampus and the cortex areas when given placebo in their diet. Comparing the placebo group of 13 months, old mice with the vitamin E treated mice from early age i.e. from 5 months until 13 months showed decrease in the induction of both the peptide forms, (Aβ 1-40 & Aβ 1-42) in high salt and formic acid soluble fractions. Conversely, there was no significant difference observed in the 14 months old mice when treated with the supplementation as compared to the placebo group. They also analysed the Aβ deposition in the brain areas (somatosensory, hippocampus, and peri-hippocampus cortex). Using immunohistochemistry, they ended with the results that thegroup treated at early age of 5 months until 13 months with the supplementation, showed significant reduction in the amyloid burden, whereas there was no significant reduction observed in the 14 months old mice as compared to the placebo treated group of both the ages. Supporting the above-mentioned statement that supplementation at early stages but not laterin the life span, (Morris et al., 2005) showed that vitamin E was able to decrease the lipid peroxidation vulnerability by 60% in the AD patients.

It has been demonstrated that in AD pathology, the accumulation of soluble Aβ and its insoluble oligomers lead to activation of microglial cells via binding to its receptors including α6β1, CD47, CD14, CD36, Toll Like Receptors (TLRs) in the CNS that leads to the release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, ultimately ending to the course of neuroinflammation, a prime biomarker of AD (Fassbender et al., 2004; Stewart et al., 2010; Berg et al., 2012; Du et al., 1997; Paresce et al., 1996; Khoury et al., 1996; Bamberger et al., 2003; Sheedy et al., 2013), wherein certain isoforms vitamin E can be hypothesized to slow the progression AD as because of its strong anti-inflammatory properties (reviewed in Singh et al., 2005; Lloret et al., 2019; Browne et al., 2019). Another study has demonstrated that inhibition of the enzyme COX-2, 5-lipooxygenase, the potent synthesizer of pro-inflammatory cytokine involved in the onset of neuroinflammation and oxidative damage during AD, can be achieved by vitamin E and related tocotrienols again because of its anti-inflammatory property (Block, 2008; Chu and Praticò, 2011; McGeer, and McGeer, 2007). Moreover, PP2A, a phosphatase involved in tau homeostasis, was activated by vitamin E action as this was found to be down regulated in the brains of patients with AD (Voronkov et al., 2011).

(Miquel, 1973) used *D. melanogaster* as the model and fed approximately 3000 adult male flies with three different concentrations of α-tocopherol acetate (0.06%,0.12% & 0.25%). Determining the life span, they achieved a significant increase in the life span of the flies with the highest concentration showing the highest beneficial effect.

(Hensley et al., 1999) explained that p38 activity is increased in the brains of patients with AD.

(Giraldo et al., 2014) in their study did both in-vitro and in-vivo experiments to determine whether oxidative stress and p38 activation are related to amyloid beta toxicity and tau hyperphosphorylation. They determined the p38 activity because p38 is a class of MAPKs which are activated in response to extracellular stimuli and are thought to get activated in response to oxidative stress, neuroinflammation, and in response to Aβ aggregates in case of AD. They did in-vitro study using foetal rat neurons in order to check whether p38 activation has relation with the Aβ toxicity and tau hyperphosphorylation, wherein they incubated the cells with Aβ and found increase in the p38 and tau phosphorylation. This increase in the activation was averted by co-incubation of these cellswith Trolox, a homologue of vitamin E. To check whether a-beta induced tau phosphorylation is due to p38 activity, they co-incubated the culture with p38 inhibitor (SB203580) before treatment with Aβ. The result showed that when p38 is inhibited, there is a decrease in the tau phosphorylation as compared to the neuros with p38 inhibitor. To confirm whether the significant results achieved in the in-vitro studies are similar in in-vivo, they utilised double transgenic mice model i.e. APP/PS1. Their studies demonstrated that AD micehippocampus showed greater levels of P-p38 as compared to the WT mice. But this condition was prevented (p38 activation) when these mice were fed with vitamin E supplemented food suggesting that Vitamin E protects against Neurofibrillary tangles of hyperphosphorylated tau, through inhibition of p38 MAPK, in animal models of vitamin E deficiency.

(Yatin et al., 2000) in their study predicted that Aβ (1-42) induced neurotoxicity in AD is a consequence of oxidative stress. To check their prediction, they compared ROS levels of untreated neuronal cells and those treated with Aβ (1-42) wherein they found low levels of ROS formation in the untreated cells but significant increase in the ROS formation was observed in the cells treated with Aβ (1-42) which was subsequently reduced when the cells were treated with vitamin E (added to rat hippocampal cells 1 hour prior to Aβ (1-42) administration). Quantifying the ROS, the data indicated that Aβ (1-42) increases the ROSformation four-fold when compared to the control. This increased ROS formation was observed to get reduced when treated with vitamin E. They also analysed the protein carbonyl levels, a by-product of protein oxidation in the cell culture, wherein they found that the cells administered with Aβ (1-42) had 168% protein carbonyl level than control, but this was suppressed to controls when the cultures were pre-treated with vitamin E.

(Wang et al., 2016) previously in their study demonstrated that using alpha-tocopherol quinine hindered Aβ accumulation and toxicity lowered down the liberation of the inflammatory cytokines and reactive oxygen species in vitro. In this study, they used α-TQ and showed that orally given α-tocopherol improved memory deficits by lowering the Aβ aggregates, lowered oxidative stress, and the release of inflammatory cytokines in vivo in transgenic mice model. In their experiment to check whether α-TQ suppresses the cognitiveimpairment in transgenic mice with AD, MWM behavioural test was conducted in which they trained mice for 5 consecutive days (training period) to find the

hidden platform and found that the α-TQ treated and the WT mice showed improved escape latency as compared to the AD control. They did this without the platform for the probe trial, wherein they found that the α-TQ treated mice with AD and the WT showed shorter escape latency as compared to the vehicle-treated mice with AD and also the number of platform crossings (during training session) was increased in the α-TQ as compared to the AD control. These results suggest that treating the transgenic mice with AD significantly improves the spatial memory. As it is known that AD neuroinflammation plays a major role in its pathogenicity, they determined whether α-TQ could attenuate the activation of microglia. They first established that the Iba1 levels were higher in mice with AD which is associated with microglial activation in comparison with the WT, and further treatment (AD mice) with α-TQ decreased the levels of Iba1 (immunohistochemistry results). To confirm, they further investigated the expression levels of Iba1, wherein they treated the cultured BV-2 cells with Aβ 42, and different concentrations of α-TQ, results established that treating the cells with α-TQ dose dependently decreases the expression levels of Iba1. Thus, these results suggest that α-TQ could attenuate the activation of microglia. They also reported that that in transgenic AD mice model, the endogenous Cu/Zn SOD levels were reduced and MDA levels were increased which is a product of lipid peroxidation where successive treatment of this groupof mice with α-TQ significantly increased the Cu/Zn SOD levels and reduced the MDA levels, suggesting a role in enhancing the endogenous antioxidant levels and function as an effective antioxidant for treatment of AD. As mentioned above, α-TQ is the oxidised form ofα-T which is the main component of vitamin E. Through redox cycling of quinine and generating of the antioxidant hydroquinone, a detoxifying function inhibiting the ROS generated in AD can be performed.

(Dias-Santagata et al., 2007) in their study to determine whether intracellularly aggregated tau in AD has a relation with enhanced oxidative stress, they did genetic manipulation of tau and modified levels of the endogenous antioxidants. In this approach, they genetically modified the flies with pan-neuronal expression of human neurodegenerative disease related mutant tau giving transgenic model (tau^{R406W}) wherein over expression of mutant tau and down regulation of endogenous SOD2, enhanced the tau toxicity increasing neurodegeneration. Upregulating the antioxidant levels by adding vitamin E in the tau mutant flies (tau^{R406W}), they demonstrated that the dose concentrations0.5Mm and 1.5Mm significantly suppressed the tau induced toxicity. Suggesting the role ofvitamin E as an antioxidant and importance of the endogenous antioxidants also, (Yang et al., 2005; Wang et al., 2006) have shown that supplementing flies with vitamin E under normal conditions does not extend the life span but significantly abandons the phenotypes ofneurodegenerative diseases of humans. Studies on *C. elegans* and mice have also illustratedthat supplementing vitamin E, extended the life spans of both the organisms (Navarro et al., 2005;Harrington and Harley, 1988). (Jhoo et al., 2004) illustrated that when Aβ (1-42) treatment was given to mice, Cu/Zn SOD activity was increased in cerebral cortex and hippocampus when compared to Aβ (40-1), suggesting a need for antioxidants to

abolish the Aβ induced toxicity. Subsequent supplementing α-tocopherol, suppressed the increase in the Cu/Zn SOD activity when compared to Aβ (1-42) non α-tocopherol treated mice. Suggesting the role α-tocopherol as a potent antioxidant for treatment of AD, this was also done to determine the role of α -tocopherol in regulating the Mn-SOD activity, wherein similar results were obtained. In a previous study, the same group found that administering A1-42 to mice continually reduced the protein expression of Mn-SOD, GSH, GPX, and glutathione-S-transferase, indicating cytological effects of the drug on the mice's brains and possibly a decline in antioxidant capability (Kim et al., 2003). However, their present study demonstrated an increase in the levels of *Cu/Zn-SOD*, *Mn-SOD*, *GPX* and *GRX* antioxidant enzyme activity in brains of an animal model, however this was not found in the group of mice treated with Aβ (40-1) (Jhoo et al., 2004). As greater number of studieshave reported that vitamin E along with its diverse role in AD has the major role as an antioxidant, few are there who have questioned the ability of vitamin E as the same of which (Giraldo et al., 2014) has defined this variability as a function of differences in the response of individuals consuming the antioxidant property of vitamin E. On this basis, they suggested that there are two types of population; one in which the antioxidant property of vitamin E is fruitful resulting in decrease of functional decline, especially in the case of AD; on the other hand a population which does not respond efficiently to this treatment. Anotherstudy done by (Yamada et al., 1999) illustrated that treating the Aβ 1-42 infused rats with idebenone and α-tocopherol, averted the behavioural deficits. The antioxidant property of vitamin E is thought to work independent of the transcriptional events. Induction of Nrf 2 longevity promoting transcription factor, having genes encoding for antioxidant proteins, was observed following treatment with vitamin E. The induction of this transcription factor by vitamin E is achieved by either increasing the Nrf2 expression levels or preventing Nrf2 inhibition by potent inhibitors (Li et al., 2012).

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgement

The authors are very much thankful to Science and Engineering Research Board (SERB), New Delhi, India (No.EMR/2016/006911/HS) and Gujarat Council of Science and Technology (GUJCOST/MRP/2015-16/2680) Gujarat, for financial support to AKT. The infrastructure support by the Puri Foundation for Education in India is duly acknowledged.

References

Adalbert, R., Milde, S., Durrant, C., Ando, K., Stygelbout, V., Yilmaz, Z., and Coleman, M. P. (2018). Interaction between a MAPT variant causing frontotemporal dementia andmutant APP affects axonal transport. *Neurobiology of aging, 68,* 68-75.

Adams, K. B., & Sanders, S. (2004). Alzheimer's caregiver differences in experience of loss,grief

reactions and depressive symptoms across stage of disease: a mixed-method analysis. *Dementia*, *3*(2), 195-210.

Adeniji, A. O., Adams, P. W., & Mody, V. V. (2017). Amyloid β hypothesis in the development of therapeutic agents for Alzheimer's disease. In *Drug Discovery Approaches for the Treatment of Neurodegenerative Disorders* (pp. 109-143). Academic Press.

Afanas' ev, I. (2011). ROS and RNS signaling in heart disorders: could antioxidant treatment be successful? *Oxidative medicine and cellular longevity*, *2011*.

Aggarwal, B. B., Sundaram, C., Prasad, S., & Kannappan, R. (2010). Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochemical pharmacology*, *80*(11), 1613-1631.

Aguilar, T. A. F., Navarro, B. C. H., & Pérez, J. A. M. (2016). Endogenous antioxidants: a review of their role in oxidative stress. *A master regulator of oxidative stress-the transcription factor nrf2*, 3-20.

Ahmad, W., Ijaz, B., Shabbiri, K., Ahmed, F., & Rehman, S. (2017). Oxidative toxicity in diabetes and Alzheimer's disease: mechanisms behind ROS/RNS generation. *Journal of biomedical science*, *24*(1), 1-10.

Ahsan, H., Ahad, A., & Siddiqui, W. A. (2015). A review of characterization of tocotrienolsfrom plant oils and foods. *Journal of chemical biology*, *8*(2), 45-59.

Akaaboune, M., Allinquant, B., Farza, H., Roy, K., Magoul, R., Fiszman, M., & Hantaï, D. (2000). Developmental regulation of amyloid precursor protein at the neuromuscularjunction in mouse skeletal muscle. *Molecular and Cellular Neuroscience*, *15*(4), 355-367. Akaike, T. (2001). Role of free radicals in viral pathogenesis and mutation. *Reviews inmedical virology*, *11*(2), 87-101.

Altomare, A., Baron, G., Gianazza, E., Banfi, C., Carini, M., & Aldini, G. (2021). Lipid peroxidation derived reactive carbonyl species in free and conjugated forms as an index of lipid peroxidation: limits and perspectives. *Redox Biology*, *42*, 101899.

Arita, M., Nomura, K., Arai, H., & Inoue, K. (1997). α-Tocopherol transfer protein stimulates the secretion of α-tocopherol from a cultured liver cell line through a brefeldin A-insensitive pathway. *Proceedings of the National Academy of Sciences*, *94*(23), 12437- 12441.

Arlt, S., Müller- Thomsen, T., & Beisiegel, U. (2002). Use of vitamin C and E in the treatmentof Alzheimer's disease. *Drug development research*, *56*(3), 452-457.

Armstrong, R. A. (2006). Plaques and tangles and the pathogenesis of Alzheimer's disease. *Folia Neuropathologica*, *44*(1), 1-11.

Atanasiu, V., Stoian, I., Cercasov, C., & Manolescu, B. (2006). alpha-tocopherol transfer protein: Molecular aspects of specific recognition of alpha-tocopherol. *Revue Roumaine de Chimie*, *51*(3),

163.

Augustyniak, A., Bartosz, G., Čipak, A., Duburs, G., Horáková, L. U., Łuczaj, W., & Žarković, N. (2010). Natural and synthetic antioxidants: an updated overview. *Free radicalresearch*, *44*(10), 1216-1262.

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative medicine and cellular longevity*, *2014*.

Azzi, A. (2019). Tocopherols, tocotrienols and tocomonoenols: Many similar molecules butonly one vitamin E. *Redox Biology*, *26*, 101259.

Bahadorani, S., Bahadorani, P., Phillips, J. P., & Hilliker, A. J. (2008). The effects of vitamin supplementation on Drosophila life span under normoxia and under oxidative stress. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *63*(1), 35-42.

Bamberger, M. E., Harris, M. E., McDonald, D. R., Husemann, J., & Landreth, G. E. (2003).A cell surface receptor complex for fibrillar β-amyloid mediates microglial activation. *Journal of Neuroscience*, *23*(7), 2665-2674.

Bando, I., Reus, M. I. S., Andrés, D., & Cascales, M. (2005). Endogenous antioxidant defence system in rat liver following mercury chloride oral intoxication. *Journal of Biochemical and Molecular Toxicology*, *19*(3), 154-161.

Barbier, P., Zejneli, O., Martinho, M., Lasorsa, A., Belle, V., Smet-Nocca, C., ... & Landrieu, I. (2019). Role of tau as a microtubule-associated protein: structural and functional aspects. *Frontiers in aging neuroscience*, *11*, 204.

Behl, C. (1999). Alzheimer's disease and oxidative stress: implications for novel therapeuticapproaches. *Progress in neurobiology*, *57*(3), 301-323.

Bell, E. F. (1987). History of vitamin E in infant nutrition. *The American journal of clinicalnutrition*, *46*(1), 183-186.

Bellows, L., Moore, R., Anderson, J., & Young, L. (2012). Fat-soluble vitamins: A, d, e, and k. *Food and nutrition series. Health; no. 9.315*.

Benítez, M. J., Cuadros, R., & Jiménez, J. S. (2021). Phosphorylation and dephosphorylationof tau protein by the catalytic subunit of PKA, as probed by electrophoretic mobility retard. *Journal of Alzheimer's Disease*, *79*(3), 1143-1156.

Birch-Machin, M. A., & Turnbull, D. M. (2001). Assaying mitochondrial respiratory complex activity in mitochondria isolated from human cells and tissues. *Methods in cell biology*, *65*, 97-117.

Birla, H., Minocha, T., Kumar, G., Misra, A., & Singh, S. K. (2020). Role of oxidative stressand metal

toxicity in the progression of Alzheimer's disease. *Current Neuropharmacology*,*18*(7), 552-562.

Block, M. L. (2008). NADPH oxidase as a therapeutic target in AD. *BMC neuroscience*, *9*(2), 1-8.

Blokhina, O., Virolainen, E., & Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of botany*, *91*(2), 179-194.

Boothby, L. A., & Doering, P. L. (2005). Vitamin C and vitamin E for Alzheimer's disease. *Annals of Pharmacotherapy*, *39*(12), 2073-2080.

Brigelius- Flohé, R., & Traber, M. G. (1999). Vitamin E: function and metabolism. *The FASEB journal*, *13*(10), 1145-1155.

Browne, D., McGuinness, B., Woodside, J. V., & McKay, G. J. (2019). Vitamin E and AD:what do we know so far? *Clinical interventions in aging*, *14*, 1303.

Burton, G., & Ingold, K. U. (1981). Autoxidation of biological molecules. 1. Antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. *Journal of the American Chemical Society*, *103*(21), 6472-6477.

Butterfield, D. A., Swomley, A. M., & Sultana, R. (2013). Amyloid β-peptide (1–42)- induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. *Antioxidants & redox signaling*, *19*(8), 823-835.

Cai, Q., & Tammineni, P. (2016). Alterations in mitochondrial quality control in Alzheimer'sdisease. *Frontiers in cellular neuroscience*, *10*, 24.

Caldwell, J. H., Klevanski, M., Saar, M., & Müller, U. C. (2013). Roles of the amyloid precursor protein family in the peripheral nervous system. *Mechanisms of development*, *130*(6-8), 433-446.

Cassidy, L., Fernandez, F., Johnson, J. B., Naiker, M., Owoola, A. G., & Broszczak, D. A. (2020). Oxidative stress in alzheimer's disease: A review on emergent natural polyphenolic therapeutics. *Complementary therapies in medicine*, *49*, 102294.

Castellani, R. J., Harris, P. L., Sayre, L. M., Fujii, J., Taniguchi, N., Vitek, M. P., & Smith, M. A. (2001). Active glycation in neurofibrillary pathology of Alzheimer disease: Nε- (carboxymethyl) lysine and hexitol-lysine. *Free Radical Biology and Medicine*, *31*(2), 175-180.

Catalá, A., & Díaz, M. (2016). Impact of lipid peroxidation on the physiology and pathophysiology of cell membranes. *Frontiers in physiology*, *7*, 423.

Cha, M. Y., Han, S. H., Son, S. M., Hong, H. S., Choi, Y. J., Byun, J., & Mook-Jung, I. (2012). Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death. *PloS one*, *7*(4), e34929.

Chen, J. X., & Yan, S. D. (2007). Amyloid-β-induced mitochondrial dysfunction. *Journal ofAlzheimer's*

Disease, *12*(2), 177-184.

Chen, Z., & Zhong, C. (2014). Oxidative stress in Alzheimer's disease. *Neuroscience bulletin*, *30*(2), 271-281.

Chen, G. F., Xu, T. H., Yan, Y., Zhou, Y. R., Jiang, Y., Melcher, K., & Xu, H. E. (2017). Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacologica Sinica*, *38*(9), 1205-1235.

Chow, V. W., Mattson, M. P., Wong, P. C., & Gleichmann, M. (2010). An overview of APP processing enzymes and products. *Neuromolecular medicine*, *12*(1), 1-12.

Chu, J., & Praticò, D. (2011). 5- lipoxygenase as an endogenous modulator of amyloid betaformation in vivo. *Annals of neurology*, *69*(1), 34-46.

Chun, J., Lee, J., Ye, L., Exler, J., & Eitenmiller, R. R. (2006). Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *Journal of FoodComposition and Analysis*, *19*(2-3), 196-204.

Constantinescu, A., Han, D., & Packer, L. (1993). Vitamin E recycling in human erythrocytemembranes. *Journal of Biological Chemistry*, *268*(15), 10906-10913.

Conti, M., Couturier, M., Lemonnier, F., & Lemonnier, A. (1990). Antioxidant properties ofvitamin E and membrane permeability in human fibroblast cultures. In *Antioxidants in therapy and preventive medicine* (pp. 125-128). Springer, Boston, MA.

Correia, S. C., Perry, G., & Moreira, P. I. (2016). Mitochondrial traffic jams in Alzheimer'sdisease-pinpointing the roadblocks. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1862*(10), 1909-1917.

Cui, X., Lin, Q., & Liang, Y. (2020). Plant-derived antioxidants protect the nervous systemfrom aging by inhibiting oxidative stress. *Frontiers in Aging Neuroscience*, *12*, 209.

Dang, S., Wu, S., Wang, J., Li, H., Huang, M., He, W., ... & Shi, Y. (2015). Cleavage of amyloid precursor protein by an archaeal presenilin homologue PSH. *Proceedings of the National Academy of Sciences*, *112*(11), 3344-3349.

Dassa, A., & Amir, D. (2014). The role of singing familiar songs in encouraging conversation among people with middle to late stage Alzheimer's disease. *Journal of musictherapy*, *51*(2), 131-153.

DeTure, M. A., & Dickson, D. W. (2019). The neuropathological diagnosis of Alzheimer'sdisease. *Molecular neurodegeneration*, *14*(1), 1-18.

Di Meo, S., Reed, T. T., Venditti, P., & Victor, V. M. (2016). Role of ROS and RNS sources in physiological and pathological conditions. *Oxidative medicine and cellular longevity*, *2016*.

Di Meo, S., & Venditti, P. (2020). Evolution of the knowledge of free radicals and otheroxidants. *Oxidative Medicine and Cellular Longevity*, *2020*.
Dias-Santagata, D., Fulga, T. A., Duttaroy, A., & Feany, M. B. (2007). Oxidative stress mediates tau-induced neurodegeneration in Drosophila. *The Journal of clinical investigation*, *117*(1), 236-245.

Dreher, M. L. (2012). Pistachio nuts: composition and potential health benefits. *Nutrition reviews*, *70*(4), 234-240.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiological reviews*.

Drouin, G., Godin, J. R., & Pagé, B. (2011). The genetics of vitamin C loss in vertebrates. *Current genomics*, *12*(5), 371-378.

Du Yan, S., Zhu, H., Fu, J., Yan, S. F., Roher, A., Tourtellotte, W. W., & Schmidt, A. M.(1997). Amyloid-β peptide–Receptor for Advanced Glycation Endproduct interaction elicitsneuronal expression of macrophage-colony stimulating factor: A proinflammatory pathway in Alzheimer disease. *Proceedings of the National Academy of Sciences*, *94*(10), 5296-5301.

Dunn, J. D., Alvarez, L. A., Zhang, X., & Soldati, T. (2015). Reactive oxygen species and mitochondria: A nexus of cellular homeostasis. *Redox biology*, *6*, 472-485.

Dyrks, T., Weidemann, A., Multhaup, G., Salbaum, J. M., Lemaire, H. G., Kang, J., & Beyreuther, K. (1988). Identification, transmembrane orientation and biogenesis of the amyloid A4 precursor of Alzheimer's disease. *The EMBO journal*, *7*(4), 949-957.

Eckert, A., Schmitt, K., & Götz, J. (2011). Mitochondrial dysfunction-the beginning of the end in Alzheimer's disease? Separate and synergistic modes of tau and amyloid-β toxicity. *Alzheimer's research & therapy*, *3*(2), 1-11.

Eddaikra, A., & Eddaikra, N. (2021). Endogenous enzymatic antioxidant defense and pathologies. In *Antioxidants-Benefits, Sources, Mechanisms of Action*. Intech Open.

Edwards, G., Olson, C. G., Euritt, C. P., & Koulen, P. (2022). Molecular Mechanisms Underlying the Therapeutic Role of Vitamin E in Age-Related Macular Degeneration. *Frontiers in Neuroscience*, *16*.

Evans, H. M., & Bishop, K. S. (1922). On the existence of a hitherto unrecognized dietary factor essential for reproduction. *Science*, *56*(1458), 650-651.

F Lichtenthaler, S. (2012). Alpha-secretase cleavage of the amyloid precursor protein: proteolysis regulated by signaling pathways and protein trafficking. *Current Alzheimer research*, *9*(2), 165-177.

Farina, N., Llewellyn, D., Isaac, M., & Tabet, N. (2017). The use of vitamin E in the treatment of mild cognitive impairment and AD (AD). *Cochrane database of systematic Review*.

Fassbender, K., Walter, S., Kühl, S., Landmann, R., Ishii, K., Bertsch, T., ... & Beyreuther, K. (2004). The LPS receptor (CD14) links innate immunity with AD. *The FASEB Journal*,*18*(1),

203-205.

Fritz, K. S., & Petersen, D. R. (2013). An overview of the chemistry and biology of reactivealdehydes. *Free Radical Biology and Medicine*, *59*, 85-91.

Frozza, R. L., Lourenco, M. V., & De Felice, F. G. (2018). Challenges for Alzheimer's disease therapy: insights from novel mechanisms beyond memory defects. *Frontiers in neuroscience*, *12*, 37.

Fu, J. Y., Che, H. L., Tan, D. M. Y., & Teng, K. T. (2014). Bioavailability of tocotrienols: evidence in human studies. *Nutrition & metabolism*, *11*(1), 1-10.

Fukai, T., & Ushio-Fukai, M. (2011). Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxidants & redox signaling*, *15*(6), 1583-1606.

Galli, F., Azzi, A., Birringer, M., Cook-Mills, J. M., Eggersdorfer, M., Frank, J., & Özer, N. K. (2017). Vitamin E: Emerging aspects and new directions. Free Radical Biology and Medicine, 102, 16-36.

Galloway, S., Jian, L., Johnsen, R., Chew, S., & Mamo, J. C. (2007). β-Amyloid or its precursor protein is found in epithelial cells of the small intestine and is stimulated by high-fat feeding. *The Journal of nutritional biochemistry*, *18*(4), 279-284.

Gan, L., Cookson, M. R., Petrucelli, L., & La Spada, A. R. (2018). Converging pathways in neurodegeneration, from genetics to mechanisms. *Nature neuroscience*, *21*(10), 1300-1309.

Gella, A., & Durany, N. (2009). Oxidative stress in Alzheimer disease. *Cell adhesion & migration*, *3*(1), 88-93.

Giraldo, E., Lloret, A., Fuchsberger, T., & Viña, J. (2014). Aβ and tau toxicities inAlzheimer's are linked via oxidative stress-induced p38 activation: protective role of vitamin E. *Redox biology*, *2*, 873-877.

Godswill, A. G., Somtochukwu, I. V., Ikechukwu, A. O., & Kate, E. C. (2020). Health benefits of micronutrients (vitamins and minerals) and their associated deficiency diseases:A systematic review. *International Journal of Food Sciences*, *3*(1), 1-32.

Gohil, K., Schock, B. C., Chakraborty, A. A., Terasawa, Y., Raber, J., Farese Jr, R. V., & Traber, M. G. (2003). Gene expression profile of oxidant stress and neurodegeneration in transgenic mice deficient in α-tocopherol transfer protein. *Free radical biology and medicine*, *35*(11), 1343-1354.

Gong, C. X., & Iqbal, K. (2008). Hyperphosphorylation of microtubule-associated protein tau: a promising therapeutic target for Alzheimer disease. *Current medicinal chemistry*, *15*(23), 2321-2328.

Gosztyla, M. L., Brothers, H. M., & Robinson, S. R. (2018). Alzheimer's Amyloid-β is an Antimicrobial Peptide: A Review of the Evidence. *Journal of Alzheimer's disease*, *62*(4), 1495-1506.

Haak, N. J. (2003). “Do You Hear What I Mean?” A Lived Experience of Disrupted Communication in Mid-to-Late Stage Alzheimer's disease. *Alzheimer's Care Today*, *4*(1), 26-40.
Hamasaki, H., Honda, H., Suzuki, S. O., Shijo, M., Ohara, T., Hatabe, Y., & Iwaki, T. (2019). Tauopathy in basal ganglia involvement is exacerbated in a subset of patients with Alzheimer's disease: the Hisayama study. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, *11*, 415-423.

Harrington, L. A., & Harley, C. B. (1988). Effect of vitamin E on lifespan and reproduction in Caenorhabditis elegans. *Mechanisms of ageing and development*, *43*(1), 71-78.

He, L., He, T., Farrar, S., Ji, L., Liu, T., & Ma, X. (2017). Antioxidants maintain cellular redox homeostasis by elimination of reactive oxygen species. *Cellular Physiology and Biochemistry*, *44*(2), 532-553.

Hensley, K., Floyd, R. A., Zheng, N. Y., Nael, R., Robinson, K. A., Nguyen, X., ... & Bing, G. (1999). p38 kinase is activated in the AD brain. *Journal of neurochemistry*, *72*(5), 2053-2058.

Herzog, V., Kirfel, G., Siemes, C., & Schmitz, A. (2004). Biological roles of APP in the epidermis. *European journal of cell biology*, *83*(11-12), 613-624.

Hippius, H., & Neundörfer, G. (2022). The discovery of Alzheimer's disease. *Dialogues in clinical neuroscience*.

Hoe, H. S., Lee, H. K., & Pak, D. T. (2012). The upside of APP at synapses. *CNS neuroscience & therapeutics*, *18*(1), 47-56.

Hopper, T., Bayles, K. A., & Kim, E. (2001). Retained neuropsychological abilities of individuals with Alzheimer's disease. In *Seminars in Speech and Language* (Vol. 22, No. 04,pp. 261-274). Copyright© 2001 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel.:+ 1 (212) 584-4662.

Howard, A. C., McNeil, A. K., & McNeil, P. L. (2011). Promotion of plasma membrane repair by vitamin E. *Nature communications*, *2*(1), 1-8.

Huang, W. J., Zhang, X. I. A., & Chen, W. W. (2016). Role of oxidative stress in Alzheimer'sdisease. *Biomedical reports*, *4*(5), 519-522.

Ibrahim, M. M., & Gabr, M. T. (2019). Multitarget therapeutic strategies for Alzheimer’s disease. *Neural regeneration research*, *14*(3), 437.

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria journal of medicine*, *54*(4), 287-293.

Irato, P., & Santovito, G. (2021). Enzymatic and non-enzymatic molecules with antioxidantfunction. *Antioxidants*, *10*(4), 579.

Isaac, M. G. E. K. N., Quinn, R., & Tabet, N. (2008). Vitamin E for Alzheimer's disease andmild cognitive impairment. *Cochrane Database of Systematic Reviews*, (3).

Jahn, H. (2022). Memory loss in Alzheimer's disease. *Dialogues in clinical neuroscience*.

Jellinger, K. A. (2010). Basic mechanisms of neurodegeneration: a critical update. *Journal of cellular and molecular medicine*, *14*(3), 457-487.

Jhoo, J. H., Kim, H. C., Nabeshima, T., Yamada, K., Shin, E. J., Jhoo, W. K., & Woo, J. I. (2004). β-Amyloid (1–42)-induced learning and memory deficits in mice: involvement ofoxidative burdens in the hippocampus and cerebral cortex. *Behavioural brain research*, *155*(2), 185-196.

Jiang, Q., Christen, S., Shigenaga, M. K., & Ames, B. N. (2001). γ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. *The American journal of clinicalnutrition*, *74*(6), 714-722.

Jiang, Q. (2022). Metabolism of natural forms of vitamin E and biological actions of vitamin E metabolites. *Free Radical Biology and Medicine*, *179*, 375-387.

Jie, C. V., Treyer, V., Schibli, R., & Mu, L. (2021). Tauvid™: The First FDA-Approved PET Tracer for Imaging Tau Pathology in Alzheimer ’s disease. *Pharmaceuticals*, *14*(2), 110.

Johannsen, D. L., & Ravussin, E. (2009). The role of mitochondria in health and disease. *Current opinion in pharmacology*, *9*(6), 780-786.

Johnson, E. J., & Mohn, E. S. (2015). Fat-soluble vitamins. In *Nutrition for the primary careprovider* (Vol. 111, pp. 38-44). Karger Publishers.

Kadavath, H., Hofele, R. V., Biernat, J., Kumar, S., Tepper, K., Urlaub, H., ... & Zweckstetter, M. (2015). Tau stabilizes microtubules by binding at the interface between tubulin heterodimers. *Proceedings of the National Academy of Sciences*, *112*(24), 7501- 7506.

Kakkar, R., Bains, J. S., & Sharma, S. P. (1996). Effect of vitamin E on life span, malondialdehyde content and antioxidant enzymes in aging Zaprionus paravittiger. *Gerontology*, *42*(6), 312-321.

Kang, J., Lemaire, H. G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K. H., & Müller-Hill, B. (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, *325*(6106), 733-736.

Kazim, S. F., & Iqbal, K. (2016). Neurotrophic factor small-molecule mimetics mediated neuroregeneration and synaptic repair: emerging therapeutic modality for Alzheimer’s disease. *Molecular neurodegeneration*, *11*(1), 1-16.

Khan, F., Garg, V. K., Singh, A. K., & Kumar, T. (2018). Role of free radicals and certain antioxidants

in the management of huntington's disease: A review. *J. Anal. Pharm. Res*, *7*, 386-392.

Khanna, R. D., Karki, K., Pande, D., Negi, R., & Khanna, R. S. (2014). Inflammation, free radical damage, oxidative stress and cancer. *Interdiscip J Microinflammation*, *1*(1), 1000109.

Khoury, J. E., Hickman, S. E., Thomas, C. A., Cao, L., Silverstein, S. C., & Loike, J. D. (1996). Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. *Nature*, *382*(6593), 716-719.

Kelleher III, R. J., & Shen, J. (2017). Presenilin-1 mutations and Alzheimer's disease. *Proceedings of the National Academy of Sciences*, *114*(4), 629-631.

Kirova, A. M., Bays, R. B., & Lagalwar, S. (2015). Working memory and executive functiondecline across normal aging, mild cognitive impairment, and Alzheimer's disease. *BioMed research international*, *2015*.

Koedam, E. L., Lauffer, V., van der Vlies, A. E., van der Flier, W. M., Scheltens, P., & Pijnenburg, Y. A. (2010). Early-versus late-onset Alzheimer's disease: more than age alone.*Journal of Alzheimer's disease*, *19*(4), 1401-1408.

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J., & Ripova, D. (2012). Structure and pathology of tau protein in Alzheimer disease. *International journal of Alzheimer's disease,2012*.

Kopeikina, K. J., Carlson, G. A., Pitstick, R., Ludvigson, A. E., Peters, A., Luebke, J. I., ... & Spires-Jones, T. L. (2011). Tau accumulation causes mitochondrial distribution deficits inneurons in a mouse model of tauopathy and in human Alzheimer's disease brain. *The American journal of pathology*, *179*(4), 2071-2082.

Korabecny, J., Spilovska, K., Soukup, O., Dolezal, R., & Kuca, K. (2018). Amyloid Beta Hypothesis: Attention to β-and γ-Secretase Modulators. In *Alzheimer's disease-The 21st Century Challenge*. Intech Open.

Kryscio, R. J., Abner, E. L., Caban-Holt, A., Lovell, M., Goodman, P., Darke, A. K., & Schmitt, F. A. (2017). Association of antioxidant supplement use and dementia in the prevention of Alzheimer's disease by vitamin E and selenium trial (PREADViSE). *JAMA neurology*, *74*(5), 567-573.

Kuchan, M. J., Moulton, C. J., Dyer, R. A., Jensen, S. K., Schimpf, K. J., & Innis, S. M. (2018). RRR-α-tocopherol is the predominant stereoisomer of α-tocopherol in human milk.*Current developments in nutrition*, *2*(8), nzy055.

Kumar, R. R., Rao, P. H., Subramanian, V. V., & Sivasubramanian, V. (2014). Enzymatic and non-enzymatic antioxidant potentials of Chlorella vulgaris grown in effluent of a confectionery industry. *Journal of food science and technology*, *51*(2), 322-328.

Lakey, P. S., Berkemeier, T., Tong, H., Arangio, A. M., Lucas, K., Pöschl, U., & Shiraiwa, M. (2016). Chemical exposure-response relationship between air pollutants and reactiveoxygen

species in the human respiratory tract. *Scientific reports*, *6*(1), 1-6.

Lee, Y. H., Tharp, W. G., Maple, R. L., Nair, S., Permana, P. A., & Pratley, R. E. (2008). Amyloid precursor protein expression is upregulated in adipocytes in obesity. *Obesity*, *16*(7),1493-1500.

Lelieveld, S., Wilson, J., Dovrou, E., Mishra, A., Lakey, P. S., Shiraiwa, M., & Berkemeier, T. (2021). Hydroxyl radical production by air pollutants in epithelial lining fluidgoverned by interconversion and scavenging of reactive oxygen species. *Environmental science & technology*, *55*(20), 14069-14079.

Leth, T., & Søndergaard, H. (1977). Biological activity of vitamin E compounds and naturalmaterials by the resorption-gestation test, and chemical determination of the vitamin E activity in foods and feeds. *The Journal of nutrition*, *107*(12), 2236-2243.

Li, D., Saldeen, T., Romeo, F., & Mehta, J. L. (1999). Relative effects of α-and γ-tocopherolon low-density lipoprotein oxidation and superoxide dismutase and nitric oxide synthase activity and protein expression in rats. *Journal of Cardiovascular Pharmacology and Therapeutics*, *4*(4), 219-226.

Li, G., Lee, M. J., Liu, A. B., Yang, Z., Lin, Y., Shih, W. J., & Yang, C. S. (2012). The antioxidant and anti-inflammatory activities of tocopherols are independent of Nrf2 in mice. *Free Radical Biology and Medicine*, *52*(7), 1151-1158.

Liebscher, S., & Meyer-Luehmann, M. (2012). A peephole into the brain: Neuropathologicalfeatures of Alzheimer's disease revealed by in vivo two-photon imaging. *Frontiers in psychiatry*, *3*, 26.

Liu, Z., Ren, Z., Zhang, J., Chuang, C. C., Kandaswamy, E., Zhou, T., & Zuo, L. (2018). Role of ROS and nutritional antioxidants in human diseases. *Frontiers in physiology*, *9*, 477.

Lloret, A., Esteve, D., Monllor, P., Cervera-Ferri, A., & Lloret, A. (2019). The effectivenessof vitamin E treatment in AD. *International journal of molecular sciences*, *20*(4), 879.

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews*, *4*(8), 118.

Lodovici, M., & Bigagli, E. (2011). Oxidative stress and air pollution exposure. *Journal of toxicology*, *2011*.

Lovell, M. A., Ehmann, W. D., Butler, S. M., & Markesbery, W. R. (1995). Elevated thiobarbituric acid-reactive substances and antioxidant enzyme activity in the brain in Alzheimer's disease. *Neurology*, *45*(8), 1594-1601.

Maes, O. C., Chertkow, H. M., Wang, E., & Schipper, H. M. (2011). Stress gene deregulationin alzheimer peripheral blood mononuclear cells. In *Studies on Experimental Models* (pp. 251-263). Humana Press, Totowa, NJ.

Manor, D., & Morley, S. (2007). The α- tocopherol transfer protein. *Vitamins & Hormones*, *76*, 45-65.

Markesbery, W. R. (1999). The role of oxidative stress in Alzheimer disease. *Archives of neurology*, *56*(12), 1449-1452.

Martemucci, G., Costagliola, C., Mariano, M., D'andrea, L., Napolitano, P., &D'Alessandro, A. G. (2022). Free Radical Properties, Source and Targets, Antioxidant Consumption and Health. *Oxygen*, *2*(2), 48-78.

Martier, R., & Konstantinova, P. (2020). Gene therapy for neurodegenerative diseases: Slowing down the ticking clock. *Frontiers in Neuroscience*, *14*, 580179.

Mazuryk, O., Stochel, G., & Brindell, M. (2020). Variations in reactive oxygen species generation by urban airborne particulate matter in lung epithelial cells—impact of inorganicfraction. *Frontiers in chemistry*, *8*, 581752.

McCay, P. B., & Poyer, J. L. (1976). Enzyme-generated free radicals as initiators of lipid peroxidation in biological membranes. In *The enzymes of biological membranes* (pp. 239- 256). Springer, Boston, MA.

McGeer, P. L., & McGeer, E. G. (2007). NSAIDs and Alzheimer disease: epidemiological,animal model and clinical studies. *Neurobiology of aging*, *28*(5), 639-647.

McLaughlin, P. J., & Weihrauch, J. L. (1979). Vitamin E content of foods. *Journal of the American Dietetic Association*, *75*(6), 647-665.

Metodiewa, D., & Kośka, C. (1999). Reactive oxygen species and reactive nitrogen species:relevance to cyto (neuro) toxic events and neurologic disorders. An overview. *NeurotoxicityResearch*, *1*(3), 197-233.

Michalak, M. (2022). Plant-derived antioxidants: Significance in skin health and the ageingprocess. *International journal of molecular sciences*, *23*(2), 585.

Mietelska-Porowska, A., Wasik, U., Goras, M., Filipek, A., & Niewiadomska, G. (2014). Tau protein modifications and interactions: their role in function and dysfunction. *International journal of molecular sciences*, *15*(3), 4671-4713.

Mijatović, S., Savić-Radojević, A., Plješa-Ercegovac, M., Simić, T., Nicoletti, F., & Maksimović-Ivanić, D. (2020). The double-faced role of nitric oxide and reactive oxygen species in solid tumors. *Antioxidants*, *9*(5), 374.

Miquel, J. (1973). Effects of DL-α-tocopherol on the life-span of Drosophila melanogaster. *Gerontologist*, *13*, 37.

Misrani, A., Tabassum, S., & Yang, L. (2021). Mitochondrial dysfunction and oxidativestress in Alzheimer's disease. *Frontiers in aging neuroscience*, *13*, 617588.

Montagna, E., Dorostkar, M. M., & Herms, J. (2017). The role of APP in structural spine plasticity.

Frontiers in molecular neuroscience, *10*, 136.

Morris, M. C., Evans, D. A., Tangney, C. C., Bienias, J. L., Wilson, R. S., Aggarwal, N. T.,& Scherr, P. A. (2005). Relation of the tocopherol forms to incident Alzheimer disease andto cognitive change. *The American journal of clinical nutrition*, *81*(2), 508-514.

Moussa, Z., Judeh, Z. M., & Ahmed, S. A. (2019). Nonenzymatic exogenous and endogenous antioxidants. *Free Radical Medicine and Biology*, 1-22.

Muck-Seler, D., Presecki, P., Mimica, N., Mustapic, M., Pivac, N., Babic, A., ... & Folnegovic-Smalc, V. (2009). Platelet serotonin concentration and monoamine oxidase typeB activity in female patients in early, middle and late phase of Alzheimer's disease. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *33*(7), 1226-1231.

Mukai, K., Kageyama, Y., Ishida, T., & Fukuda, K. (1989). Synthesis and kinetic study of antioxidant activity of new tocopherol (vitamin E) compounds. *The Journal of Organic Chemistry*, *54*(3), 552-556.

Mukai, K., Tokunaga, A., Itoh, S., Kanesaki, Y., Ohara, K., Nagaoka, S. I., & Abe, K. (2007).Structure− activity relationship of the free-radical-scavenging reaction by vitamin E (α-, β-,γ-, δ-Tocopherols) and ubiquinol-10: pH dependence of the reaction rates. *The Journal of Physical Chemistry B*, *111*(3), 652-662.

Münch, G., Schinzel, R., Loske, C., Wong, A., Durany, N., Li, J. J., & Riederer, P. (1998).Alzheimer's disease–synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. *Journal of neural transmission*, *105*(4), 439-461.

Munné-Bosch, S., & Alegre, L. (2002). The function of tocopherols and tocotrienols in plants. *Critical Reviews in Plant Sciences*, *21*(1), 31-57.

Murpy, M., & LeVine III, H. (2010). Alzheimer's disease and the β-amyloid peptide. *J Alzheimers Dis*, *19*(1), 311-323.

Namazi, M. R. (2009). Cytochrome-P450 enzymes and autoimmunity: expansion of the relationship and introduction of free radicals as the link. *Journal of autoimmune diseases*, *6*(1), 1-5.

Navarro, A., Gómez, C., Sánchez-Pino, M. J., González, H., Bández, M. J., Boveris, A. D., & Boveris, A. (2005). Vitamin E at high doses improves survival, neurological performance,and brain mitochondrial function in aging male mice. *American Journal of Physiology- Regulatory, Integrative and Comparative Physiology*, *289*(5), R1392-R1399.

Nelson, P. T., Braak, H., & Markesbery, W. R. (2009). Neuropathology and cognitive impairment in Alzheimer disease: a complex but coherent relationship. *Journal of Neuropathology & Experimental Neurology*, *68*(1), 1-14.

Niki, E. (2007). Tocopherylquinone and tocopherylhydroquinone. *Redox Report*, *12*(5), 204-210.

Niki, E. (2014). Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro andin vivo evidence. *Free Radical Biology and Medicine*, *66*, 3-12.

Niki, E. (2015). Evidence for beneficial effects of vitamin E. *The Korean journal of internalmedicine*, *30*(5), 571.

Niki, E., & Abe, K. (2019). Vitamin E: Structure, properties and functions.

Nimse, S. B., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC advances*, *5*(35), 27986-28006.

Nunomura, A., Castellani, R. J., Zhu, X., Moreira, P. I., Perry, G., & Smith, M. A. (2006). Involvement of oxidative stress in Alzheimer disease. *Journal of neuropathology & experimental neurology*, *65*(7), 631-641.

O'brien, R. J., & Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer'sdisease. *Annual review of neuroscience*, *34*, 185-204.

O'Neill, C., Anderton, B., Brion, J. P., Anderton, B. H., Authelet, M., Dayanandan, R., ... &Tremp, G. (2001, February). Neurofibrillary tangles and tau phosphorylation. In *Biochemical Society Symposia* (Vol. 67, pp. 81-88). Portland Press.

Ozcan, A., & Ogun, M. (2015). Biochemistry of reactive oxygen and nitrogen species. *Basicprinciples and clinical significance of oxidative stress*, *3*, 37-58.

Packer, L., Weber, S. U., & Rimbach, G. (2001). Molecular aspects of α-tocotrienolantioxidant action and cell signalling. *The Journal of nutrition*, *131*(2), 369S-373S.

Padayatty, S. J., & Levine, M. (2016). Vitamin C: the known and the unknown and Goldilocks. *Oral diseases*, *22*(6), 463-493.

Paresce, D. M., Ghosh, R. N., & Maxfield, F. R. (1996). Microglial cells internalizeaggregates of the AD amyloid β-protein via a scavenger receptor. *Neuron*, *17*(3), 553-565.

Pavlik, V. N., Doody, R. S., Rountree, S. D., & Darby, E. J. (2009). Vitamin E use is associated with improved survival in an Alzheimer's disease cohort. *Dementia and geriatriccognitive disorders*, *28*(6), 536-540.

Petersen, R. C. (2017). Free-radicals and advanced chemistries involved in cell membrane organization influence oxygen diffusion and pathology treatment. *AIMS biophysics*, *4*(2), 240.

Phaniendra, A., Jestadi, D. B., & Periyasamy, L. (2015). Free radicals: properties, sources, targets, and their implication in various diseases. *Indian journal of clinical biochemistry*, *30*(1), 11-26.

Planche, V., Manjon, J. V., Mansencal, B., Lanuza, E., Tourdias, T., Catheline, G., & Coupé,

P. (2022). Structural progression of Alzheimer's disease over decades: the MRI stagingscheme. *Brain Communications*, *4*(3), fcac109.

Pluta, R., Furmaga-Jabłońska, W., Maciejewski, R., Ułamek-Kozioł, M., & Jabłoński, M. (2013). Brain ischemia activates β-and γ-secretase cleavage of amyloid precursor protein: significance in sporadic Alzheimer's disease. *Molecular neurobiology*, *47*(1), 425-434.

Praticò, D. (1999). F2-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation in vivo. *Atherosclerosis*, *147*(1), 1-10.

Praticò, D. (2002). Lipid peroxidation and the aging process. *Science of Aging Knowledge Environment*, *2002*(50), re5-re5.

Pryor, W. A., Cornicelli, J. A., Devall, L. J., Tait, B., Trivedi, B. K., Witiak, D. T., & Wu, M. (1993). A rapid screening test to determine the antioxidant potencies of natural andsynthetic antioxidants. *The Journal of Organic Chemistry*, *58*(13), 3521-3532.

Pryor, W. A., Strickland, T., & Church, D. F. (1988). Comparison of the efficiencies of several natural and synthetic antioxidants in aqueous SDS [sodium dodecyl sulfate] micellesolutions. *Journal of the American Chemical Society*, *110*(7), 2224-2229.

Puig, K. L., & Combs, C. K. (2013). Expression and function of APP and its metabolites outside the central nervous system. *Experimental gerontology*, *48*(7), 608-611.

Radi, R. (2018). Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proceedings of the National Academy of Sciences*, *115*(23), 5839-5848.

Raji, C. A., Lopez, O. L., Kuller, L. H., Carmichael, O. T., & Becker, J. T. (2009). Age, Alzheimer disease, and brain structure. *Neurology*, *73*(22), 1899-1905.

Ranard, K. M., & Erdman Jr, J. W. (2018). Effects of dietary RRR α-tocopherol vs all- racemic α-tocopherol on health outcomes. *Nutrition reviews*, *76*(3), 141-153.

Rao, Y. L., Ganaraja, B., Murlimanju, B. V., Joy, T., Krishnamurthy, A., & Agrawal, A. (2022). Hippocampus and its involvement in Alzheimer's disease: a review. *3 Biotech*, *12*(2), 1-10.

Reboul, E. (2017). Vitamin E bioavailability: mechanisms of intestinal absorption in the spotlight. *Antioxidants*, *6*(4), 95.

Reitz, C., Rogaeva, E., & Beecham, G. W. (2020). Late-onset vs nonmendelian early-onsetAlzheimer disease: A distinction without a difference? *Neurology Genetics*, *6*(5).

Repetto, M., Semprine, J., & Boveris, A. (2012). Lipid peroxidation: chemical mechanism,biological implications and analytical determination. *Lipid peroxidation*, *1*, 3-30.

Rice-Evans, C., Halliwell, B., Lunt, G. G., & Tyrrell, R. M. (1995, November). Ultraviolet radiation and free radical damage to skin. In *Biochemical society symposia* (Vol. 61, pp. 47-53). Portland Press.

Rink, C., & Khanna, S. (2011). Significance of brain tissue oxygenation and the arachidonicacid cascade in stroke. *Antioxidants & redox signaling*, *14*(10), 1889-1903.

Ristori, E., Donnini, S., & Ziche, M. (2020). New insights into blood-brain barrier maintenance: the homeostatic role of β-Amyloid precursor protein in cerebral vasculature. *Frontiers in Physiology*, *11*, 1056.

Rizvi, S., Raza, S. T., Ahmed, F., Ahmad, A., Abbas, S., & Mahdi, F. (2014). The role of vitamin E in human health and some diseases. *Sultan Qaboos University Medical Journal*, *14*(2), e157.

Salvadores, N., Gerónimo-Olvera, C., & Court, F. A. (2020). Axonal degeneration in AD: the contribution of Aβ and Tau. *Frontiers in Aging Neuroscience*, *12*, 581767.

Samanta, S., Ramesh, M., & Govindaraju, T. (2022). Alzheimer's is a Multifactorial Disease.

Selkoe, D. J., Podlisny, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C., & Oltersdorf, T. (1988). Beta-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues. *Proceedings of the National Academy of Sciences*, *85*(19), 7341-7345.

Semchyshyn, H. M. (2014). Reactive carbonyl species in vivo: generation and dual biological effects. *The Scientific World Journal*, *2014*.

Sen, C. K., Khanna, S., & Roy, S. (2006). Tocotrienols: Vitamin E beyond tocopherols. *Lifesciences*, *78*(18), 2088-2098.

Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor perspectives in medicine*, *1*(1), a006189.

Shahidi, F., & De Camargo, A. C. (2016). Tocopherols and tocotrienols in common and emerging dietary sources: Occurrence, applications, and health benefits. *International journal of molecular sciences*, *17*(10), 1745.

Shan, X. (2005). *Investigation of mRNA oxidation in Alzheimer's disease*. The Ohio State University.

Sharifi-Rad, M., Anil Kumar, N. V., Zucca, P., Varoni, E. M., Dini, L., Panzarini, E., ... & Sharifi-Rad, J. (2020). Lifestyle, oxidative stress, and antioxidants: Back and forth in the pathophysiology of chronic diseases. *Frontiers in physiology*, *11*, 694.

Sharma, C., & Kim, S. R. (2021). Linking oxidative stress and proteinopathy in Alzheimer'sdisease. *Antioxidants*, *10*(8), 1231.

Sheedy, F. J., Grebe, A., Rayner, K. J., Kalantari, P., Ramkhelawon, B., Carpenter, S. B., ...& Moore, K. J. (2013). CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nature immunology*, *14*(8), 812-820.

Sheikh, S., Haque, E., & Mir, S. S. (2013). Neurodegenerative diseases: multifactorial conformational diseases and their therapeutic interventions. *Journal of neurodegenerative diseases*, *2013*.

Shields, H. J., Traa, A., & Van Raamsdonk, J. M. (2021). Beneficial and detrimental effectsof reactive oxygen species on lifespan: A comprehensive review of comparative and experimental studies. *Frontiers in Cell and Developmental Biology*, *9*, 628157.

Siddiqui, M. A., Ahmad, U., Ali, A., Ahsan, F., & Haider, M. F. (2021). Role of Vitamin Ein Pregnancy. In *Vitamin E in Health and Disease-Interactions, Diseases and Health Aspects*. IntechOpen.

SIMAN, R. (1994). Beta-amyloid precursor: a neuronal and glial-derived protein with trophic-like activities. In *Trophic Regulation of the Basal Ganglia* (pp. 329-344). Pergamon.

Simpson, D. S., & Oliver, P. L. (2020). ROS generation in microglia: understanding oxidative stress and inflammation in neurodegenerative disease. *Antioxidants*, *9*(8), 743.

Singh, U., Devaraj, S., & Jialal, I. (2005). Vitamin E, oxidative stress, and inflammation. *Annual review of nutrition*, *25*, 151.

Singh, V. K., Beattie, L. A., & Seed, T. M. (2013). Vitamin E: tocopherols and tocotrienolsas potential radiation countermeasures. *Journal of radiation research*, *54*(6), 973-988.

Singh, S. S., & Jois, S. D. (2018). Homo-and heterodimerization of proteins in cell signaling:inhibition and drug design. *Advances in protein chemistry and structural biology*, *111*, 1-59.

Skender-Gazibara, M., & Slobodan, D. O. Æ. I. (2001). Neuropathological hallmarks of Alzheimer's disease. *Arch Oncol*, *9*(1), 5-7.

Small, D. H., & Cappai, R. (2006). Alois Alzheimer and Alzheimer's disease: a centennial perspective. *Journal of neurochemistry*, *99*(3), 708-710.

Sontheimer, H. (2015). Aging, Dementia, and Alzheimer. *Diseases of the Nervous System*, *99*.

Stevens, S. L. (2021). Fat-soluble vitamins. *Nursing Clinics*, *56*(1), 33-45.

Stewart, C. R., Stuart, L. M., Wilkinson, K., Van Gils, J. M., Deng, J., Halle, A., & Moore, K. J. (2010). CD36 ligands promote sterile inflammation through assembly of a Toll-likereceptor 4 and 6 heterodimer. *Nature immunology*, *11*(2), 155-161.

Stocker, A., & Azzi, A. (2000). Tocopherol-binding proteins: their function and physiological

significance. *Antioxidants and Redox Signaling*, *2*(3), 397-404.

Stoia, M., & Oancea, S. (2022). Low-Molecular-Weight Synthetic Antioxidants: Classification, Pharmacological Profile, Effectiveness and Trends. *Antioxidants*, *11*(4), 638.

Sun, X., Chen, W. D., & Wang, Y. D. (2015). β-Amyloid: the key peptide in the pathogenesis of Alzheimer's disease. *Frontiers in pharmacology*, *6*, 221.

Sung, S., Yao, Y., Uryu, K., Yang, H., Lee, V. M. Y., Trojanowski, J. Q., & Praticò, D. (2004). Early vitamin E supplementation in young but not aged mice reduces Aβ levels andamyloid deposition in a transgenic model of AD. *The FASEB journal*, *18*(2), 323-325.

Szewczyk, K., Chojnacka, A., & Górnicka, M. (2021). Tocopherols and tocotrienols— Bioactive dietary compounds; what is certain, what is doubt? *International Journal of Molecular Sciences*, *22*(12), 6222.

Szymanska, R., Pospisil, P., & Kruk, J. (2016). Plant-derived antioxidants in diseaseprevention. *Oxidative medicine and cellular longevity*, *2016*.

Szymanska, R., Pospíšil, P., & Kruk, J. (2018). Plant-derived antioxidants in disease prevention 2018. *Oxidative medicine and cellular longevity*, *2018*.

Tabet, N., & Birks, J. (2000). Vitamin E for Alzheimer's disease. *The Cochrane Databaseof Systematic Reviews*, (4), CD002854-CD002854.

Tagarelli, A., Piro, A., Tagarelli, G., Lagonia, P., & Quattrone, A. (2006). Alois Alzheimer:a hundred years after the discovery of the eponymous disorder. *International journal of biomedical science: IJBS*, *2*(2), 196.

Takashima, A. (2016). Mechanism of neurodegeneration through Tau and therapy for Alzheimer's disease. *Journal of sport and health science*, *5*(4), 391.

Tamagno, E., Guglielmotto, M., Vasciaveo, V., & Tabaton, M. (2021). Oxidative Stress andBeta Amyloid in Alzheimer's disease. Which Comes First: The Chicken or the Egg? *Antioxidants*, *10*(9), 1479.

Tardy, A. L., Pouteau, E., Marquez, D., Yilmaz, C., & Scholey, A. (2020). Vitamins and minerals for energy, fatigue and cognition: a narrative review of the biochemical and clinical evidence. Nutrients, 12(1), 228.

Theriault, A., Chao, J. T., Wang, Q. I., Gapor, A., & Adeli, K. (1999). Tocotrienol: a reviewof its therapeutic potential. *Clinical biochemistry*, *32*(5), 309-319.

Thiele, J. J., Schroeter, C., Hsieh, S. N., Podda, M., & Packer, L. (2001). The antioxidant network of the stratum corneum. *CURRENT PROBLEMS IN DERMATOLOGY-BASEL-*, *29*,26-42.

Thinakaran, G. (1999). The role of presenilins in Alzheimer's disease. *The Journal of clinical investigation*, *104*(10), 1321-1327.

Thomas, D. R. (2006). Vitamins in aging, health, and longevity. *Clinical interventions in aging*, *1*(1), 81.

Tillement, L., Lecanu, L., & Papadopoulos, V. (2011). Alzheimer's disease: effects of β- amyloid on mitochondria. *Mitochondrion*, *11*(1), 13-21.

Tönnies, E., & Trushina, E. (2017). Oxidative stress, synaptic dysfunction, and Alzheimer'sdisease. *Journal of Alzheimer's disease*, *57*(4), 1105-1121.

Tvrdá, E., Massanyi, P., & Lukáč, N. (2017). Physiological and pathological roles of free radicals in male reproduction. In *Spermatozoa-Facts and Perspectives*. London, UK: IntechOpen.

Ulatowski, L., Ghelfi, M., West, R., Atkinson, J., Finno, C. J., & Manor, D. (2022). The tocopherol transfer protein mediates vitamin E trafficking between cerebellar astrocytes and neurons. *Journal of Biological Chemistry*, *298*(3).

Uzombah, T. A. (2022). The Implications of Replacing Synthetic Antioxidants with NaturalOnes in the Food Systems.

Vadas, D., Kalichman, L., Hadanny, A., & Efrati, S. (2017). Hyperbaric oxygen environmentcan enhance brain activity and multitasking performance. *Frontiers in integrative neuroscience*, *11*, 25.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1), 44-84.

Vatassery, G. T., Smith, W. E., Quach, H. T., & Lai, J. C. K. (1995). In vitro oxidation of vitamin E, vitamin C, thiols and cholesterol in rat brain mitochondria incubated with free radicals. *Neurochemistry international*, *26*(5), 527-535.

Veith, A., & Moorthy, B. (2018). Role of cytochrome P450s in the generation and metabolism of reactive oxygen species. *Current opinion in toxicology*, *7*, 44-51.

Vom Berg, J., Prokop, S., Miller, K. R., Obst, J., Kälin, R. E., Lopategui-Cabezas, I., ... & Heppner, F. L. (2012). Inhibition of IL-12/IL-23 signaling reduces AD–like pathology and cognitive decline. *Nature medicine*, *18*(12), 1812-1819.

Voronkov, M., Braithwaite, S. P., & Stock, J. B. (2011). Phosphoprotein phosphatase 2A: anovel druggable target for AD. *Future medicinal chemistry*, *3*(7), 821-833.

Wallert, M., Kluge, S., Schubert, M., Koeberle, A., Werz, O., Birringer, M., & Lorkowski, S. (2020). Diversity of chromanol and chromenol structures and functions: An emerging class of anti-

inflammatory and anti-carcinogenic agents. *Frontiers in Pharmacology*, *11*, 362.

Wang, D., Qian, L., Xiong, H., Liu, J., Neckameyer, W. S., Oldham, S., & Zhang, Z. (2006). Antioxidants protect PINK1-dependent dopaminergic neurons in Drosophila. *Proceedings of the National Academy of Sciences*, *103*(36), 13520-13525.

Wang, S. W., Yang, S. G., Liu, W., Zhang, Y. X., Xu, P. X., Wang, T., ... & Liu, R. T. (2016). Alpha-tocopherol quinine ameliorates spatial memory deficits by reducing beta- amyloid oligomers, neuroinflammation and oxidative stress in transgenic mice with AD. *Behavioural brain research*, *296*, 109-117.

Wang, X., & Quinn, P. J. (1999). Vitamin E and its function in membranes. *Progress in lipidresearch*, *38*(4), 309-336.

Wang, X., & Quinn, P. J. (2000). The location and function of vitamin E in membranes. *Molecular membrane biology*, *17*(3), 143-156.

Wang, X., Su, B. O., Siedlak, S. L., Moreira, P. I., Fujioka, H., Wang, Y., ... & Zhu, X. (2008). Amyloid-β overproduction causes abnormal mitochondrial dynamics via differentialmodulation of mitochondrial fission/fusion proteins. *Proceedings of the National Academy of Sciences*, *105*(49), 19318-19323.

Webster, N. R., & Nunn, J. F. (1988). Molecular structure of free radicals and their importance in biological reactions. *British journal of anaesthesia*, *60*(1), 98-108.

Wong, R. S., & Radhakrishnan, A. K. (2012). Tocotrienol research: past into present. *Nutrition reviews*, *70*(9), 483-490.

Xu, T. H., Yan, Y., Kang, Y., Jiang, Y., Melcher, K., & Xu, H. E. (2016). Alzheimer's disease-associated mutations increase amyloid precursor protein resistance to γ-secretase cleavage and the Aβ42/Aβ40 ratio. *Cell Discovery*, *2*(1), 1-14.

Yamada, K., Tanaka, T., Han, D., Senzaki, K., Kameyama, T., & Nabeshima, T. (1999). Protective effects of idebenone and α- tocopherol on β- amyloid- (1–42) - induced learning andmemory deficits in rats: implication of oxidative stress in β- amyloid- induced neurotoxicity in vivo. *European Journal of Neuroscience*, *11*(1), 83-90.

Yang, Y., Wu, Z., Kuo, Y. M., & Zhou, B. (2005). Dietary rescue of fumble—a Drosophila model for pantothenate-kinase-associated neurodegeneration. *Journal of inherited metabolicdisease*, *28*(6), 1055-1064.

Yaseen, A. A., Al-Okbi, S. Y., Hussein, A. M., Mohamed, D. A., Mohammad, A. A., Fouda, K. A., & Mehaya, F. M. (2019). Potential protection from Alzheimer's disease by wheat germ and rice bran nano-form in rat model. *Journal of Applied Pharmaceutical Science*, *9*(1), 067-076.

Yatin, S. M., Varadarajan, S., & Butterfield, D. A. (2000). Vitamin E prevents Alzheimer'samyloid ß-Peptide (1-42)-induced neuronal protein oxidation and reactive oxygen species production. *Journal of AD*, *2*(2), 123-131.

Yuste, J. E., Tarragon, E., Campuzano, C. M., & Ros-Bernal, F. (2015). Implications of glialnitric oxide in neurodegenerative diseases. *Frontiers in cellular neuroscience*, *9*, 322.

Zarkovic, N. (2020). Roles and Functions of ROS and RNS in Cellular Physiology and Pathology. *Cells*, *9*(3), 767.

Zhang, X., & Song, W. (2013). The role of APP and BACE1 trafficking in APP processingand amyloid-β generation. *Alzheimer's research & therapy*, *5*(5), 1-8.

Zhang, Y. W., Thompson, R., Zhang, H., & Xu, H. (2011). APP processing in Alzheimer's disease. *Molecular brain*, *4*(1), 1-13.

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species(ROS) and ROS-induced ROS release. *Physiological reviews*, *94*(3), 909-950.

Review Article

Bacteria as emerging indicators of soil condition

Rajkishan Sinh Thakor[a], Vishwa Patel[a], Dhyey Bhatt[a], Hillery Gandhi[a], and Shuvomoy Banerjee[a*]

[a] *Bioinformatics Laboratory, School of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institutional Area, Gandhinagar 382426, India*

**Corresponding author: shuvomoy.banerjee@iar.ac.in*

Abstract

The production of high-quality, nutrient-dense food requires healthy soil, which is crucial today due to the massive use of chemical fertilisers that may have dangerous effects on human health as well as detrimental effects on soil quality. As a result, it is crucial to understand the quality of the soil and how to improve it. There are various indicators of soil health (Nielsen and Winding, n.d.). There are some biological indicators which indicate the nutrient value of the soil like soil microbial protein, organic matter, and respiration. The amount of CO2 produced by microorganisms during respiration can be used to determine the level of microbial activity in the soil, and the diversity of the bacteria present in the soil is another important indicator (*The Biogeography of Stream Bacteria - Lear - 2013 - Global Ecology and Biogeography - Wiley Online Library*, n.d.). Soil microorganisms decompose and transfer the organic materials which are derived fromabove and below-ground plant residues. Microbes present in the soil play a crucial role in carbon cycling, nutrient turnover, or the production of trace gases (*Microbiome of the Upper Troposphere: Species Composition and Prevalence, Effects of Tropical Storms, and Atmospheric Implications |PNAS*, n.d.). It is well-known that soil microbial activities, populations, and communities are governed by environmental variables such as soil type and texture, temperature, moisture, or pH (Hermans et al., 2017).

Introduction

Healthy soil is the main factor for survival on earth and it is essential for the integrity of terrestrial ecosystems. There are some disturbances such as drought, climate change, pest infestation, pollution, and human exploitation including agriculture which reduce the quality of soil, so protection of soil is high priority nowadays (*Comparison of Soil Bacterial Communities under Diverse Agricultural Land Management and Crop Production Practices - PubMed*, n.d.). There is no proper definition for soil quality standards like air and water. Water and air quality based on the maximum allowable concentration of materials is hazardous to human health. A definition of soil health based on this concept would encompass on a fraction of many roles soil plays, to manage and maintain soil sustainably; therefore, the definition of soil health must be broad enough to encompass many functions of soil, e.g. environmental filter, plant growth, and water regulation (*The Diversity and Biogeography of Soil Bacterial*

Communities | PNAS, n.d.). The need for a systematic approach to protect soil ecosystems within Europe has been described in the draft report of the Sixth Environmental Action Program "Environment 2010: Our future, Our Choice", which was presented by the European Commission in the beginning of 2001. A European monitoring and assessment framework on soil has subsequently been proposed to provide policy-makers with relevant information on soil and to bring together the wealth of soil information derived from current national soil monitoring programmes (*Biofilms in Fresh water: Their Importance for the Maintenance and Monitoring of Freshwater Health*, n.d.).

The impact of land use change and management on soil microbial community composition is poorly understood. Therefore, the scientists have explored the relationship between a wide range of soil bacterial community composition and soil factors (*Monitoring Complex Bacterial Communities Using Culture-Independent Molecular Techniques: Application to Soil Environment PubMed*, n.d.). The research has provided the evidence of strong relationships between individuqaal tax and specific soil attributes even across large spatial scales and soil and land use types. Collectively, they were able to demonstrate the largely untapped potential of micro organisms to indicate the conditions of soil and thereby influence the way that monitored the effects of anthropogenic activity on soil ecosystems into the future. Soil bacterial communities provide a multitude of ecosystem services which directly and indirectly affect the overall functioning of the soil environment (*Use of Plant Growth-Promoting Bacteria for Biocontrol of Plant Diseases: Principles, Mechanisms of Action, and Future Prospects | Applied and Environmental Microbiology*, n.d.). This has resulted in many studies describing variations in bacterial communitycomposition and functional roles. There is great promise for using bacterial community as indicators of soil condition. Microbial community indicators can offer significant advantages over traditional chemical and biological measures in terms of different dynamics. The relationships between specific soil attributes and individual soil taxanomy not only highlights ecological characteristics of these organisms, but also demonstrates the ability of key bacterial taxonomic groups to reflect the impact of specific anthropogenic activities (*Monitoring Complex Bacterial Communities Using Culture-Independent Molecular Techniques: Application to Soil Environment PubMed*, n.d.).

Soil is the most important ecosystem component which has the ability to maintain quality of surrounding air and water and its quality is defined by how it is supporting plant and animal productivity (*Heavy Metals in Agricultural Soils of the European Union with Implications for Food Safety – Science Direct*, n.d.). High-quality soils are therefore crucial for sustaining agricultural and pastoral industries upon which both food security and financial stability depend. Soils has a diversity of microbial life which contribute to the cycling of important nutrients, impact plant growth, and can act as or protect other organisms from pathogens (*Bacterial and Fungal Communities in Bulk Soil and Rhizospheres of Aluminum-Tolerant and Aluminum-Sensitive Maize(Zea Mays L.) Lines Cultivated in Unlimed and Limed Cerrado Soil - PubMed*, n.d.). Microorganisms interact with microorganisms to facilitate the decomposition process. Despite the importance of living organisms for maintaining healthy soil ecosystems, most initiatives that directly monitor soil quality for applied purposes focus on changes in

abiotic variables such as soil nutrients, metal pollutants and soil structure (*Influence of Soil Characteristics on the Diversity of Bacteria in the Southern Brazilian Atlantic Forest - PubMed*, n.d.). Where biological measures are included in monitoring efforts, they are often crude and generalized such as microbial biomass or soil respiration, although some use more specific organisms such as earthworms, as more sensitive indicators (*A Review of Soil Quality Indicators and Five Key Issues after 12 Yr Soil Quality Monitoring in the Waikato Region - Taylor - 2010 - Soil Use and Management - Wiley Online Library*, n.d.). Better incorporation of biological indicators in soil monitoring will provide a more sensitive, relevant, and holistic insight into how anthropogenic activity impacts the soil environment (*A Review of Soil Quality Indicators and Five Key Issues after 12 Yr Soil Quality Monitoring in the Waikato Region - Taylor - 2010 - Soil Use and Management - Wiley Online Library*, n.d.).

Soil bacterial communities are strongly impacted by changes in soil conditions. The diversity and composition of bacterial communities change with changing soil acidity. Large-scale predictions of bacterial diversity are possibly based on pH data alone (Nitrogen Fixation by Rhizobium Cultured on a Defined Medium Nature, n.d.). Additionally, plant diversity, nutrient concentrations, soil moisture and soil type have all been shown to correlate with changes in bacterial communities. Importantly, there is evidence that bacterial communities directly or indirectly respond to changes in the soil environment brought on by the anthropogenic activity. Overall, the composition of bacterial communities appear to be heavily influenced by changes in the soil environment, many of which are the direct result of land use activities (Lauber et al., 2013).

There are many statistical methods available for indicator development based on bacterial community data; particularly promising are machine learning approaches. Broadly speaking, these involve creating a predictive model through identifying discriminating independent variables; if successful, the model can then be used to classify new samples from an assessment of the biological data (Lauber et al., 2013).

Importance of soil indicators

Soil can be considered as hotspot for microbial biodiversity on earth due to their often large and complex microbiomes. As a result, soils provide a large number of biological services that are essential for life on earth which are known as Life Support Functions (LSF) (*The Bacterial Biogeography of British Soils – Pub Med*, n.d.). As the studies show, soil has multifunctionality whichis highly endangered as a result of the on-going global change. Thus, the persistent threat of soil degradation driven by climatic and anthropogenic forces prioritizes the development of strict directives for the protection of soil. The importance of developing robust, reliable, and resilient biological indicators for monitoring the soil quality has been increased in order to establish a warning system of potential losses of the multi functionality of soils (*Comparative Metagenomic, Phylogenetic and Physiological Analyses of Soil Microbial Communities across Nitrogen Gradients| The ISME Journal*, n.d.).

About soil indicators

Such indicators should be measured easily and should be accurate for the purpose they were developed for. In addition, it would be advantageous if the costs were kept low (*Reconstructing the Microbial Diversity and Function of Pre-Agricultural Tallgrass Prairie Soils in the United States - PubMed*, n.d.).

In the past, several efforts have been made to define biological indicators of soil quality which mainly focused on the 'visible parts' of the soil biota but the currently existing indicators are mostly based on so called black box parameters which are microbial biomass, global or potential microbial activity patterns, or assays that determine potential enzymatic activities (*A Novel Bacterial Community Index to Assess Stream Ecological Health - Lau - 2015 - FreshwaterBiology - Wiley Online Library*, n.d.). A series of experiments were conducted to assess differentrelationships of different factors with the bacteria.

Relationships among spatial variables, soil factors, and bacterial community composition at the regional scale

The linear regression analysis proved that generally the five different categories of land uses showed similarity in results in terms of the relationship between bacterial community dissimilarity and geographic distance, climatic dissimilarity, or dissimilarity with geographic distance. An important relationship between bacterial community dissimilarity and changes in climate was observed only for dairy and dry stock sites, indicating that overall, this was not an important variable affecting the bacterial communities at the scale of the sample collected (*The Influence of Soil Properties on the Structure of Bacterial and Fungal Communities across Land-Use Types – Science Direct*, n.d.). Similarly, for most of the land uses, the increase in bacterial dissimilarity with increasing geographic distance was minimal.

Relationship between bacterial community composition and soil parameters

Overall, pH, carbon-nitrogen (C: N) ratio, and Olsen P (plant scale available phosphorus) was responsible for greater variation in bacterial community composition than other variables. Especially distance-based multi-variate multiple regression showed that pH accounted for the largest number of variations in community composition, especially in relation to the members of Planctomycetes (*Three Common Metal Contaminants of Urban Runoff (Zn, Cu & Pb) Accumulate in Freshwater Biofilm and Modify Embedded Bacterial Communities – Science Direct*, n.d.).

Relationships within individual anthropogenic land uses

If the bacterial indicators are used to inform the soil condition under-managed land uses, then important relationships between key taxanomy and attributes should remain, even when soil under native land use is excluded from the analysis.

Horticulture soil had significantly higher soil pH than most of the other land uses but the dairy farming soil was an exception. Even if limiting the study to solely data from horticulture sites, dairy sites, or native forest sites, members of the family Pirelluaceae were co-related with this soil measure in addition to these variations in soil pH across land uses.The indigenous forest sites on average had higher C: N than other sites. The genus *Bradyrhizobium* was well co-related with the level of Olsen P in horticulture soils. The *Chitinophagaceae* group was co-related with concentration of aluminum within land uses.

References

Ancion, P.Y., Lear, G., Lewis, G.D., Three common metal contaminants of urban runoff (Zn, Cu & Pb) accumulate in freshwater biofilm and modify embedded bacterial communities, Environmental Pollution, 2010,158(8),2738-2745.

Compant, S., Duffy, B., Nowak, J., Clément, C., Barka, E.A., Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol. 2005 Sep; 71(9):4951-9.

Da Mota, F.F., Gomes, E.A., Marriel, I.E., Paiva, E., Seldin, L., Bacterial and fungal communities in bulk soiland rhizospheres of aluminum-tolerant and aluminum-sensitive maize (Zea mays L.) lines cultivated in unlimed and limed Cerrado soil. J Microbiol Biotechnol. 2008 May; 18(5):805-14. PMID: 18633275.

DeLeon-Rodriguez, N., Lathem, T.L., Rodriguez-R, L.M., Barazesh, J.M., Anderson, B.E., Beyersdorf, A.J., Ziemba, L.D., Bergin, M., Nenes, A., Konstantinidis, K.T, Microbiome of the upper troposphere: species composition and prevalence, effects of tropical storms, and atmospheric implications. Proc Natl AcadSci U S A. 2013 Feb 12; 110(7):2575-80.

Faoro, H., Alves, A.C., Souza, E.M., Rigo, L.U., Cruz, L.M., Al-Janabi, S.M., Monteiro, R.A., Baura, V.A., Pedrosa, F.O., Influence of soil characteristics on the diversity of bacteria in the Southern Brazilian Atlantic Forest. Appl Environ Microbiol. 2010 Jul; 76(14):4744-9.

Fierer, N., Jackson, R.B., The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci U S A. 2006 Jan 17; 103(3):626-31.

Fierer, N., Ladau, J., Clemente, J.C., Leff, J.W., Owens, S.M., Pollard, K.S., Knight, R., Gilbert, J.A., McCulley, R.L., Reconstructing the microbial diversity and function of pre-agricultural tallgrass prairie soils in theUnited States. Science. 2013 Nov 1; 342(6158):621-4.

Fierer, N., Lauber, C., Ramirez, K. et al. Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 6, 1007–1017 (2012)

Griffiths, R.I., Thomson, B.C., James, P., Bell, T., Bailey, M., Whiteley, A.S., The bacterial biogeography of British soils. Environ Microbiol. 2011 Jun; 13(6):1642-54.

Hermans, S.M., Buckley, H.L., Case, B.S., Curran-Cournane, F., Taylor, M., Lear, G., Bacteria as Emerging Indicators of Soil Condition. Appl Environ Microbiol. 2016 Dec 15; 83(1):e02826-16.

Lau, KEM, Washington, V.J., Fan, V., Neale, M.W., Lear, G., Curran, J, et al. A novel bacterial communityindex to assess stream ecological health. Freshwater Biol. 2015; 60: 1988–2002.

Lauber, C., Ramirez, K., Aanderud, Z. et al. Temporal variability in soil microbial communities across land-use types. ISME J 7, 1641–1650 (2013).

Lauber, C.L., Strickland, M.S., Bradford, M.A., Fierer, N., The influence of soil properties on the structure of bacterial and fungal communities across land-use types, Soil Biology and Biochemistry,2008,40(9),2407-2415.

Lear, G., Dopheide, A., Ancion, P., Roberts, K., Washington, V., Smith, J and Lewis, GD from: Microbial Biofilms: Current Research and Applications (Edited by: Gavin Lear and Gillian D. Lewis). Caister Academic Press, U.K. (2012)

Pagan, J., Child, J., Scowcroft, W. et al. Nitrogen fixation by Rhizobium cultured on a defined medium.

Ranjard, L., Poly, F., Nazaret, S. Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment. Res Microbiol. 2000 Apr;151(3):167-77. doi: 10.1016/s0923-2508(00)00136-4. PMID: 10865943

Taylor, M.D., Kim, N.D., Hill, R.B. and Chapman, R. (2010), A review of soil quality indicators and five key issues after 12yr soil quality monitoring in the Waikato region. Soil Use and Management, 26: 212-224

Tóth, G., Hermann, T., Da Silva, M.R., Montanarella, L., Heavy metals in agricultural soils of the European Union with implications for food safety. Environ Int. 2016 Mar; 88:299-309. doi: 10.1016/j.envint.2015.12.017. Epub 2016 Feb 3. PMID: 26851498.

Wu, T., Chellemi, D.O., Graham, J.H., Martin, K.J., Rosskopf, E.N., Comparison of soil bacterial communitiesunder diverse agricultural land management and crop production practices. Microb Ecol. 2008 Feb; 55(2):293-310. Nature 256, 406–407 (1975)

Review Article

Global burden of antimicrobial drug resistance: Would sitting on a fence serve us all?

Dr. Shubhita Tripathi

Department of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institutional Area, Gandhinagar 382426, India

**Corresponding Author: E-mail: shubhita.tripathi@iar.ac.in*

Abstract

The very first step to building an effective policy to combat against the rapidly evolving AMR is to build a knowledge database that helps understand global burden of AMR and identify and target the highest priority pathogens in different locations. Although there are a plethora of scientific publicationsand reports studying AMR in different geolocations and pertaining to specific AMR causing pathogensin those locations, it is a herculean task to build a complete picture of AMR globally and to come up with key pathogens that need to be targeted. Even more efforts are required at the communityas well as individual level to combat AMR in India. As of now, substantial and exhaustive data on AMR update in India is extremely fragmented based on regional studies and/or high priority pathogens in those regions/states/health-centres etc. and does not provide a comprehensive picture. Additionally, easy access to over-the-counter antibiotics without prescription and failure to complete treatment regimens remain a major cause of concern that over time paves the way for development ofnew resistant genes imparting AMR in pathogens. Another concern especially attributable to developing countries like India is the presence of drug resistant pathogens in the ICUs that cause nosocomial infections due to the lack of hygiene in our health-care facilities. The timing of empirical antibiotic therapy in severe infections like Sepsis complicates our efforts in limiting drug resistance. On top of all this, the recent COVID-19 pandemic might have severe implications for the emergence of drug resistance globally due to high rates of inappropriate antimicrobial prescribing, the high use ofbiocides, and the interruption of treatment for other conditions. A fresh wave of efforts is required to combat the growing severity of AMR with multifaceted approaches.

Introduction

One of the leading public healthcare concerns that have been emerging as a global threat of the 21st century is the emergence and rapid evolution of antimicrobial resistance. Antimicrobial resistance (AMR), by definition, as per the world health organization (WHO) occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to medicines, making infections harder totreat and increasing the risk of disease spread, severe illness, and death. As a result of drug resistance, antibiotics and other antimicrobial medicines become ineffective and infections become increasingly difficult or impossible to treat.

As per a review commissioned by the UK government, AMR could kill over 10 million people by theyear 2050 (O'Neill, 2014 & 2016). Numerous other groups including WHO have realised and raised concerns over the emerging and rapidly evolving AMR across the globe (WHO, Geneva, 2015; US CDC, Atlanta, 2019; Prestinaci et al., 2015). AMR does occur naturally over time. This can be attributed to antimicrobial drug resistant organisms that are found in humans, animals, plants, and the environment which spread over time as a result of human-to-human transmission, animal-to-human transmission as well as from meat and other animal-based products. However, the misuse and/or overuse of antimicrobials, poor infection control and disease prevention policies in healthcare facilities, lack of affordable and quality medicines, vaccines and diagnostics etc. hastens the process of otherwise naturally occurring antimicrobial resistance. This leads to rapid emergence and spread of drug resistant pathogens quickly across the globe that have acquired new resistance mechanisms. Notably, in the last few years, the overuse and inefficient use of antimicrobials has also paved the way for emergence and global spread of multi-drug resistant and pan-drug resistant bacteria, commonly known as "Superbugs". These have caused even greater chaos and put undue pressure on the otherwise overwhelmed healthcare facilities as they are not treat able byany existing antimicrobial drugs.

Reduced efficacy of antimicrobials can impede the most important medical advances and undo decadesof milestones achieved in treating infections, managing chronic diseases, and surgical/post-surgical care. In addition to the loss of lives, AMR can also result in loss of global GDP by 2-3.5%, valued at USD 100 trillion by 2050 (Jasovsky et al., 2016; WHO, 2016). Easy access to over-the-counter antibiotics, inappropriate use of antibiotics in human beings in livestock, poor hygiene in healthcare facilities, and failure to adhere to treatment regimens are major factors leading to rapid AMR.

The global perspective

The rapidly evolving AMR is a ticking time bomb for global healthcare and can potentially disrupt decades of ground-breaking efforts contributed by scientists and healthcare organisations globally.WHO in 2017 published a list of priority pathogens that were responsible for majority cases of AMR globally. The list included the top drug-pathogen combinations that were seen as the most critical combinations fuelling AMR and responsible for rendering the most serious cases of drug resistance that were becoming extremely difficult to treat because such pathogen strains had become resistant to even the last generation antibiotics. The seven drug-pathogen combinations, as listed in the WHO priority report were namely, Methicillin-resistant *Staphylococcus aureus* (MRSA), Isoniazid and Rifampicin co-resistant (excluding XDR) *Mycobacterium tuberculosis*, Third-generation cephalosporin-resistant *Escherichia coli*, Carbapenem-resistant *Acinetobacter baumannii*, Fluoroquinolone-resistant *Escherichia coli*, Carbapenem-resistant *Klebsiella pneumonia*, and Third- generation cephalosporin-resistant *Klebsiella pneumonia*e. Although, the list was intended to realise the most difficult to treat bacterial infections but it did not define the top priority pathogens (in the order of priority) worldwide that were sounding the alarm on global AMR.

(Naghavi et al., 2019) (Figure 1) in association with a joint UK commission report published exhaustive findings on the global burden of AMR and the top priority pathogens specific for each region. The pathogens responsible were listed based on the data for deaths attributable and/or associated with resistance. Figure 1 shows top six priority pathogens that were responsible for global spread of AMR namely, *Acinetobacter*

baumannii, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumonia.* The figure also shows the top priority pathogens responsible for majority deaths attributable to resistance (Figure1a) and associated with resistance (Figure 1b).

The Indian perspective

In 2011, the government of India formulated the national policy for containment of AMR in India. As part of the directives, a detailed policy map was chalked out which aimed at capturing the incidence of 'use and misuse of antibiotics in the country' through creation of national surveillance system for antibiotic resistance, mechanism of monitoring prescription audits, diagnostic tools for AMR monitoring, regulatory provision for monitoring use of antibiotics in human, veterinary and industrialsectors and identification of specific intervention measures for rational use of antibiotics and antibiotic policies in hospitals (Govt. of India policy on AMR, 2011).

Several incidents have since been reported as novel cases of AMR that have been observed and reported as part of the national surveillance on AMR in India. The most popular example is that of the NDM-1 enzyme (New-Delhi Metallo β-Lactamase-1) that was first reported in a Swedish patient who had undergone surgery in a New-Delhi based hospital. A recent article published in Lancet (Laxminarayan et al., 2015) reported that among neonates in India, 56,254 deaths per year were caused due to sepsis resulting from a drug-resistant pathogen infection.

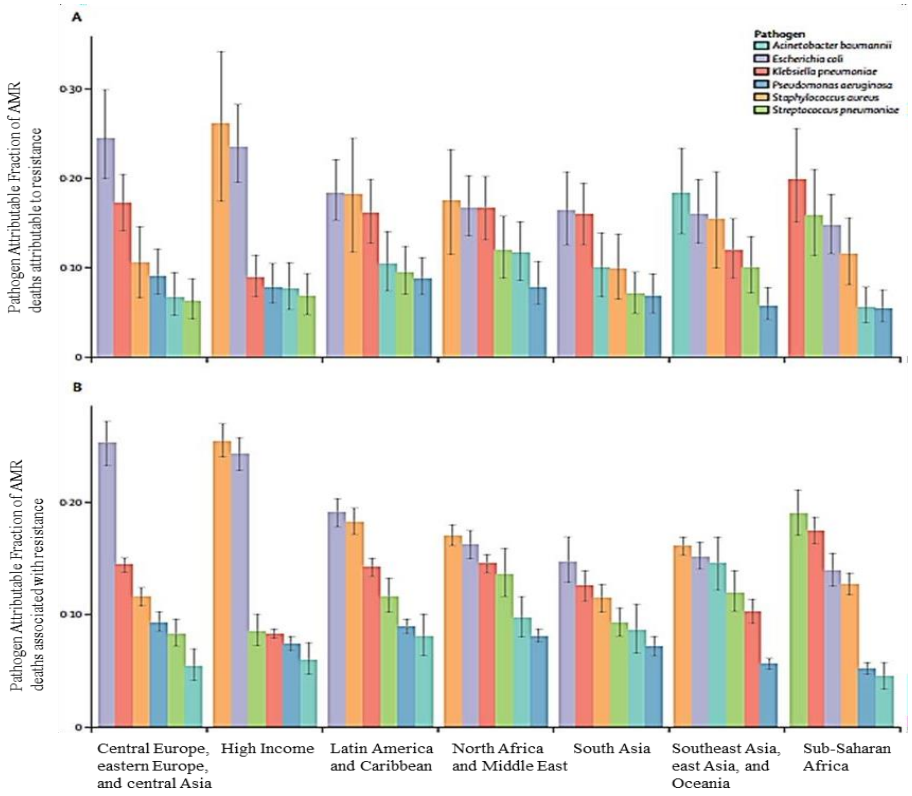


Figure 1: Major pathogens responsible for deaths attributable to resistance

In its efforts to contain the spread of AMR, the government of India in association with WHO in 2019, published its list of priority pathogens from medium to critical priority (Figure 2) based on identification of the most common bacterial infections and their resistance mechanisms from collated data provided by national experts from varied backgrounds like infectious diseases, clinical microbiology, R&D, infection prevention and control (healthcare associated infections), public health, paediatric, and intensive care.

Although recent institutional and policy-level initiatives have sparked a fresh wave of motivation to draft a new national action programme aimed at identifying novel mutant genes, resistance strains, and mechanisms of resistance as part of surveillance as well as establishing efficient therapeutic procedures to better treat resistant infections in patients, there are still a multitude of challenges that need to be overcome for us to be able to make effective progress at the ground level. The major bottlenecks in our race to combat AMR have been discussed further.

Easy access to antibiotics

Overuse and misuse of antibiotics in humans, animals, etc in India has been a major reason for the development of extended drug resistance rapidly. The availability of over-the-counter antibiotics havealso helped to escalate the situation even further. Inappropriate antibiotic therapy in the form of non-critical prophylactic antibiotic therapy and use of pan-spectrum antibiotics has helped to drive resistance mechanisms in bacteria. To worsen the situation, non-completion of a full antibiotic coursealso fuels new resistance mechanisms as sublethal doses help the pathogen to gain mutations to evadedeath. Apart from human infections, antibiotics are also used in growth promotion in animals to keep them disease-free and this helps to spread resistant strains in the environment. A recent study reportedthat irrational and overuse of antibiotics in different sectors in India have led to the emergence of extended antimicrobial resistance wherein the environment acts as a reservoir of antibiotic resistance genes (ARGs); completing the cycle of contamination and recontamination (Jani et al., 2021).

ICUs: The hot bed of drug resistance

Life-threatening infections like septic shock resulting from sepsis are common in the ICUs. Sepsis patients need rapid emergency care and it has been widely reported that lack of appropriate antibiotictherapy in less than 24 hours could result in mortality. As the risks run high, the clinicians often rely on early empirical antibiotic therapy based on the symptoms presented by the patient while waiting onthe blood culture and antibiotic sensitivity test report (AST). In addition to this, the timing of the antibiotic therapy also impacts survival. As the early empirical therapy can help build resistance withinthe infecting pathogen, subsequent specific antibiotic therapy post AST report needs to be aggressive (Kollef et al., 2021). This leads to build-up of AMR in the nosocomial strains. The next set of patients often catch these highly resistant nosocomial strains during their hospital stay and the spead of AMR within the community is carried forward.

Implications of COVID-19 pandemic

As a side-effect of the recent COVID-19 pandemic, there has been concern within the scientific community as well as the clinicians about the impact of overuse of antibiotics as prophylactic therapy. Some have suggested that this might have fuelled the start of another pandemic; one with thechronic use of antibiotics

potentially resulting in acquisitions of novel resistance genes in the common bacterial pathogens (Huttner et al., 2020). Although co-infection with COVID-19 has been a rare phenomenon (Karami et al., 2021) mostly observed in geriatric population and immuno deficient and comorbid individuals, an increased administration of antibiotics have been reported in such patients (Rawson et al., 2020). Altered pharmacodynamic properties need to be taken into account in COVID-19 infected patients prior to administration of antibiotic therapy. Future studies need to be done to assess the total impact of excessive use of antibiotics during this pandemic to better understand its implications.

CRITICAL PRIORITY	
Klebsiella pneumoniae and ***Escherichia coli***	Carbapenem-R Tigecycline-R Colistin-R
Acinetobacter baumannii and ***Pseudomonas aeruginosa***	Carbapenem-R Colistin-R
HIGH PRIORITY	
Staphylococcus aureus	MRSA, hVISA, Daptomycin-NS Linezolid-R
Enterococcus sp.	Vancomycin-R Linezolid-R Daptomycin-NS
Salmonella sp. (typhoidal and non-typhoidal)	Azithromycin-NS Third generation cephalosporin-NS Carbapenem- NS
MEDIUM PRIORITY	
Streptococcus pneumoniae	Cephalosporin-R Linezolid-R Flouroquinolone-R
Staphyloccoccus, coagulase-negative	Vancomycin-R Linezolid-R
Shigella sp.	Third-generation cephalosporin-R Azithromycin-R
Heamophilus influenzae	Third-generation cephalosporin-R Carbapenem-NS
Neisseria meningitidis	Flouroquinolone-NS Third-generation cephalosporin-NS

Figure 2: India Priority Pathogen list
(R: resistant; NS: non-susceptible; MRSA: methicillin resistant *Staph. aureus*; hVISA: heterogenous vancomycin-intermediate *Staph. Aureus)*

Ways forward

The misuse of antibiotic in different sectors in India viz. human infections, animals, and the environment are responsible for rapidly evolving resistance mechanisms in pathogens and has fuelledthe way to spread AMR globally. Effective strategies are required at the institutional as well as the community level to combat

this growing resistance (Figure 3). Several strategies like antimicrobial stewardship in the ICUs can help define proper methodologies for optimal prescription of antibiotics.This would help to limit unnecessary dosage of antibiotics in patients and limit the development of novel resistance mechanisms. Further, rapid tools to reliably detect antimicrobial resistance are the need of the hour to avoid inappropriate use of antibiotics as empirical therapy.

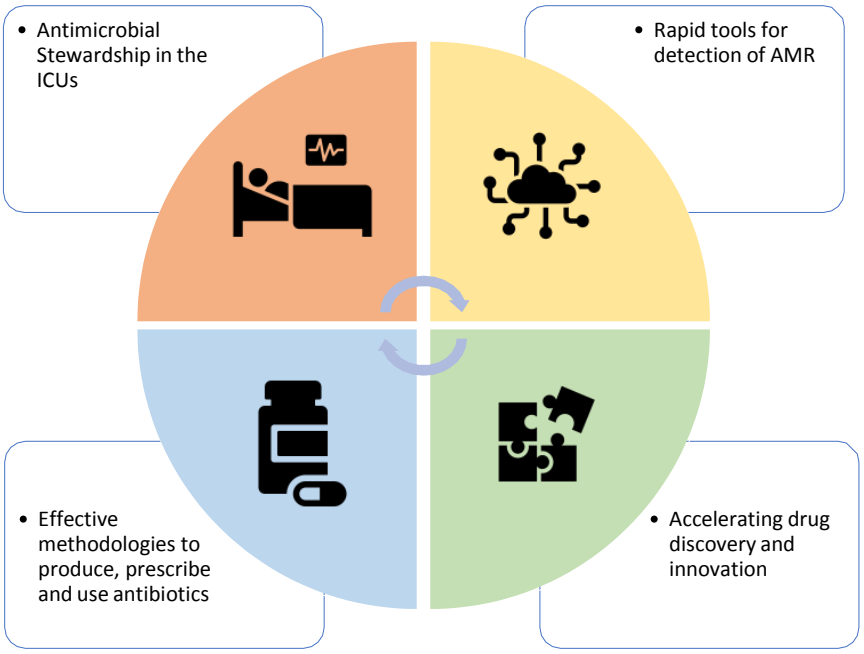


Figure 3: Effective strategies to combat antimicrobial resistance

Effective methodologies to produce novel antibiotics and better policies for effective prescription andappropriate use of antibiotics need to be implemented at the healthcare institution level under the national action programmes to limit spread of AMR.

Lastly, accelerating discovery of new antibiotics that can work on resistant pathogens like "superbugs"needs to be encouraged. A fresh wave of policy making and funding is required in this area at the international level to combat global spread of AMR and innovation and development of novel antibiotics.

References

de Kraker, MEA., Lipsitch, M. Burden of antimicrobial resistance: compared to what? Epidemiol Rev 2021; published online March 12. https://doi.org/10.1093/epirev/mxab001

Huttner, B.D., Catho, G., Pano-Pardo, J.R., Pulcini, C., Schouten, J. COVID-19: don't neglect antimicrobial stewardship principles! Clin Microbiol Infect. 2020.

Jani, K., Srivastava, V., Sharma, P., Vir, A., Sharma, A. Easy Access to Antibiotics; Spread of Antimicrobial Resistance and Implementation of One Health Approach in India. J Epidemiol Glob Health. 2021.

Jasovský, D., Jasper, L., Anna, Z and Otto, C. Antimicrobial resistancea threat to the world's sustainable development. Upsala J Med Sci. 2016; 121(3):159-64.

Kollef, M.H., Shorr, A.F., Bassetti, M. *et al*. Timing of antibiotic therapy in the ICU. *Crit Care* 25, 360. 2021

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Røttingen, J.A., Klugman, K, et al. Access to effectiveantimicrobials - a worldwide challenge. Lancet. 2015; 387:168-75 India Priority Pathogent list, DBT, Govt. of India, 2019.

O'Neill, J. Tackling drug-resistant infections globally: final report and recommendations. London: Review on Antimicrobial Resistance, 2016.

O'Neill, J. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. London: Review on Antimicrobial Resistance, 2014.

Prestinaci, F., Pezzotti, P., Pantosti, A. Antimicrobial resistance: a global multifaceted phenomenon. Pathog Glob Health 2015.

Rawson, T.M., Moore, LSP, Zhu, N., Ranganathan, N., Skolimowska, K., Gilchrist, M., Satta, G., Cooke, G., Holmes, A. Bacterial and Fungal Coinfection in Individuals with Coronavirus: A Rapid Review to Support COVID-19 Antimicrobial Prescribing. Clin Infect Dis. 2020.

WHO. Global action plan on antimicrobial resistance. Geneva: World Health Organization, 2015.

World Health Organisation. National action for global change on antimicrobial resistance, 2016. Directorate General of Health Services, Ministry of Health and Family Welfare. National Policy for Containment of Antimicrobial Resistance in India. New Delhi: Government of India, 2016 .

US Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2019. Atlanta, GA: US Department of Health and Human Services, 2019.

Zara, K., Bram, T., Anton, S., M. Dofferhoff et al. Few bacterial co-infections but frequent empiric antibiotic use in the early phase of hospitalized patients with COVID-19: results from a multicentre retrospective cohort study in The Netherlands, Infectious Diseases 2021.

Review Article

Diagnostic application of MRI for Alzheimer's disease

Jahnvi Rajput, Krina Patel, Mahisha Patel, Bhargavi Korat, Juni Banerjee*

Department of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institutional Area, Gandhinagar 382426, India

**Corresponding Author email: juni.banerjee@iar.ac.in*

Abstract

Around 55 million people worldwide have dementia, with a majority of them living in low and middle income countries. Alzheimer's disease contributes to about 60-70% cases of global dementia cases. As of now, AD has no permanent cure and the best hope lies in its targeted therapies as well as early detection. The advancement of medical imaging technologies has proved a boon for the diagnosisof various diseases including neurodegenerative diseases. Among all the imaging modalities that are in huge demand, Magnetic Resonance Imaging (MRI) has been very crucial for Alzheimer's disease.The neuroanatomical configuration and the minute structural differences of brain is hard to be inspected visually, but MRI has potential of creating the 3D-model construction of any individual AD brain. This review highlights the working principle, specific applications, and advancements of MRI in the detection of Alzheimer's disease (AD).

Keywords: Alzheimer's disease, amyloid plaques, neurofibrillary tangles, apolipoprotein E, magnetic resonance imaging, grey matter, medical imaging

1. Introduction

The human brain, which is the center of the nervous system is said to be one of the most integral and complex parts of the body. Our brain is made up of more than 100 billion neurons cells that communicate via synapses and carry out crucial functions of thinking, learning, voluntary movements,emotional responses, memory formation, etc. Apart from that, glial cells comprise a major part of the human brain. Hence, any abnormal behavior or functioning of the neurons, glial cells, synapses of thebrain may cause a major collapse of the constitutive body functionalities. One such abnormal event is the aggregation of amyloid and tau protein in the brain, which gives rise to the disability of neurons, glial cells and synaptic functions to act in a normal manner, leading to Alzheimer's disease [1].

1.1 The interplay of amyloid-beta and tau in Alzheimer's disease

In simple terms, Alzheimer's disease is the most common form of dementia. It is a brain disorder thatas previously mentioned, is formed by the deposition of two key proteins: amyloid and tau. Both of the proteins are present in healthy brains and function normally but conversely, in Alzheimer's disease,they function abnormally. The interplay of amyloid-beta and tau is very fascinating in case of AD.

Several studies clearly described apolipoprotein E ε4 isoform acting as a link between amyloid-beta and tau. Research has also proved the impairment of synapse-related gene transcription by the interplay between amyloid-beta and tau. Moreover, independent factors like common upstream triggers of abnormalities for amyloid-beta and tau abnormalities and de-regulated immune systems have been indicated in amyloid-beta and tau pathologies leading to AD. Interestingly, amyloid-beta- immunotherapy has shown a reduction of tau levels along with the amyloid-beta levels in-vivo as well as in clinical trials.

Amyloid and Tau work in a co-operative manner towards AD. Amyloid deposition leads to the formation of plaques outside the nerve cells, whereas tau deposition forms tangles inside them. Theseplaques and tangles cause damage to the nerve cells causing them to die. Eventually, the brain starts shrinking because of neuronal death. The function of a neuron is to transmit messages between different parts of the brain and from the brain to respective organs in the body [19]. Initially, such damage takes place in the memory creating parts of the brain such as the hippocampus and entorhinalcortex. Later on, the effect can be observed in the cerebral cortex areas that are responsible for language, social behavior, and reasoning [1].

1.2 Major influencing factors of Alzheimer's disease aging factors

1.2.1 Aging factors

The major factor influencing Alzheimer's disease (AD) is aging, so basically, AD is an aging related neurodegenerative disease [15]. Statistical data indicates that after 65 years of age, AD incidences double every 5 years and above 85 years of age, almost half of the population show AD. One of the concepts provide a potential mechanism related to aging. It states that free radicals (reactive oxygen species) produced during cellular respiration may play an important role in the aging process and also the development of AD [8] [9].

1.2.2 Genetic factors

Most people with Alzheimer's have the late onset form of the disease in which symptoms become apparent in their mid-60s or later. Researchers have not found a specific gene that directly causes late onset Alzheimer's, but having a form of the Apolipoprotein E (APOE) gene increases a person's risk of AD. This gene has several forms including APOE ε4, which shows an association with an earlier age of AD onset and potential risks for a person to develop AD. However, carrying the APOE ε4 form of the gene does not mean that a person will definitely develop the disease, and some people with no APOE ε4 may also develop Alzheimer's.

Scientists have also identified several regions of interest in the genome (an organism's complete set ofDNA) that may increase or decrease a person's risk for late onset Alzheimer's to varying degrees. Early onset Alzheimer's occurs between a person's 30s and mid 60s and represents less than 10% of all people with Alzheimer's. Some cases are caused by an inherited change in one of the three genes. Forothers, research shows that other genetic components are involved.

The genetic factor for AD is the point mutations in the gene coding for β-amyloid precursor protein (APP) on chromosome 21 [5]. These mutations are sufficient to cause early onset autosomal dominant familial AD. Some mutations can increase the production of β-amyloid whereas few mutations could favor the formation of long forms of β-amyloid which aggregates more readily than the short forms [6].

Moreover, most people with Down syndrome develop Alzheimer's. This may be because people with Down syndrome have an extra copy of chromosome 21, which contains the gene that generates harmfulamyloid. Hence, more research is required to find out specific genes associated with AD.

1.2.3 Environmental factors

Environmental factors also tend to play a role in the etiology of AD. A hypothesis that AD may represent a chronic active inflammatory disease was supported by the findings of (Rapoport et al., 1991) by giving evidence for mild active inflammation complement activation and the presence of inflammatory cytokines in the brain of AD patients. Also, it is believed that the recruitment and activation of the microglial cells are closely related to maturation of plaques in elderly patients [11]. A minor contribution to the burden of AD risk is a history of severehead trauma which could result in unconsciousness [13] [14].

1.3 Symptoms of AD

Alzheimer's is a progressive neurodegenerative disease that produces a wide range of symptoms. Theearly signs of the disease include forgetting recent conversations or events. As the disease progresses, it will develop severe memory impairment and lose the ability to carry out regular tasks. Medicationscan only temporarily improve or slow the progression of the symptoms. In advanced stages of the disease, complications such as dehydration, malnutrition, or infection arising due to loss of brain function could even result in death.

Medical imaging techniques

Scientific and medical imaging that provide detailed anatomic and physiologic images of parts or whole body has played a key role in the study of Alzheimer disease (AD) over the past few decades. Medical imaging includes Ultrasonography, Radiography, Computed Tomography (CT), Positron Emission Tomography (PET) and Magnetic Resonance Imaging (MRI).

2. Magnetic resonance imaging (MRI)

The MRI or nuclear magnetic resonance imaging (NMRI) is a non-invasive medical imaging technique that is used in radiology to visualize the internal structure and function of the body. MRI is used especially in musculoskeletal neurological (brain), oncological (cancer), and cardiovascular imaging.The best acquisition of the brain can be obtained from the MRI because of its high contrast, excellentspatial resolution, and high availability. MRI image is taken as input to proceed for diagnosis.

3.1 Principle and working mechanism of MRI

Our body is majorly constituted of water and thus contains hydrogen atoms. These hydrogen atoms are aligned by the powerful magnetic field which is produced by the magnets in the MRI unit without causing any chemical reaction in the body. When the magnetic field is removed, the hydrogen atoms return to their usual alignment. Now, this would emit a certain amount of energy. The energy emitted as a result of this phenomenon will largely depend on the type of body tissue. The MR scanner capturesthe energy released and creates a picture of the scanned tissue based on the information. Earlier MRI was known as NMRI where N stands for nuclear. The word nuclear was associated with ionizing radiation exposure. Hence, it is now referred to as MRI. The MRI involving a powerful magnetic field,radio frequency pulse, and computer

produces a detailed picture of soft tissues, organs, bones, and otherinternal body structures. The traditional MRI unit is a large cylindrical tube like structure that is surrounded by a circular magnet. What distinguishes MRI from CT scans and conventional X-rays is that MRI does not utilize ionizing radiation. Image registration is an important step in MRI that involvesalignment of multiple images to check for the precise differences which are not visible to the naked eye. The images used for the alignment could be taken at different times from different viewpoints orby using different imaging techniques. Another important task about image and signal processing is image de-noising for which wavelet transformation is used. Using the set of MR slices, the 3D reconstruction of the brain is obtained whose main advantage is that each slide can be viewed and examined separately for AD related brain damage. For this, segmentation process is utilized to identifythe grey matter and the white matter and calculate their respective volumes. The computation of the grey matter in the human brain is achieved automatically by the use of software programs; nowadays, one such example is Voxel-based morphometry (VBM) which is used for the clinical procedure with less execution time and applicable to any part of the brain. Finally, for the decision process, the calculated ratio of grey matter to white matter is compared to the grey matter to white matter ratio of a normal brain (the ratio is already fixed for all age groups). For any calculated grey matter to white matter ratio greater than the pre-fixed normal ratio, brain abnormality will be detected.

3.2 Application of MRI for AD

MRI is applied in neurosciences research for neurodegenerative diseases like Alzheimer's disease in clinical hospitals and research laboratories. The detection of Alzheimer's disease is possible by grey matter volume loss in the Mild cognitive impairment group than in normal-aged people. Earlier detection of AD is also possible from the data of MRI. Visual inspection is very difficult to identify the minute changes in the human brain. This could be overcome by the use of MRI 3D segmentation of the brain which would help a physician to have more information from a different perspective on AD [1].

Patients with AD have a problem with cerebral blood flow, meaning they have decreased blood flow in comparison to normal people of their age. This can be detected by perfusion imaging and arterial spin labelling. Moreover, 4D flow MRI technique is used to measure blood flow amount and the direction of its flow by analyzing the wall shear stress. The diffusion-weighted MRI (DWI) which usesdifferences in water molecular diffusion patterns is applied to reveal microscopic details about tissue architecture. The DTI (diffusion tensor imaging) is used for mapping white matter tractography.

Application of MRI is getting more attention because, unlike a PET scan that involves an injection of a very small dose of radioactive substance intravenously, MRI images are made without using any ionizing radiation. Even when compared to the applications of CT scan, an MRI can take images of any body part and in any direction to provide better soft tissue contrast by differentiating between fat,water, muscle, and other soft tissues.

3. Current and future perspective of MRI on AD

Researchers are constantly trying to develop advancements in MRI techniques to assess the process and even quantify some aspects of Alzheimer's disease. Nanoparticles used in the diagnosis and targeted drug delivery of Alzheimer's disease can be easily observed through MRI. US food and drug administration have

approved Iron Oxide nanoparticles to be used as a contrast agent. Iron Oxide has a long half-life that can covalently bind with drugs and antibodies which can increase contrast duringimaging with MRI. Similarly, monocrystalline iron oxide nanoparticles and ultra-super magnetic ironoxide nanoparticles are MRI contrast agents that are used for in-vivo detection in a transgenic mouse model.

Dysfunction of the cerebral waste clearance system (glymphatic or intramural periarterial drainage) contributing to Amyloid-β accumulation and toxic plaques can be studied with the help of non-contrast enchanced (non-CE) MRI techniques. A non-invasive but quantitative modality with the ability to identify and track many aspects of AD pathology is Saturation Transfer (ST) Magnetic Resonance Imaging (MRI). Even trial designs are currently taking the help of imaging technologies to measure the effects of a therapy on fibrillary amyloid on atrophy and metabolism by MRI and fMRI respectively.

4. Conclusion

Effective identification and diagnosis of Alzheimer's disease (AD), which is defined by the accumulation of pathogenic protein deposits, neuronal and glial degeneration, synaptic loss, and synaptic dysfunctions, is essential.Although there are many known imaging methods for the detection of AD, MRI contributes to effective, relatively cheap, and universal imaging modalities that require low expertise. MRI being a great modality to quantify brain and subcortical volumes, is widely used inassessment of suspected AD. There is a certain threshold value set for the ratio of grey volume to whitevolume. If it exceeds the threshold, then it is detected as an abnormality. The basic rule of MRI analysis for AD shows a ratio of grey matter to the white matter as 1.1 for a normal 20-45 years aged person, 1.3 for a 50-80-year-old, and 1.5 for more than 60 years of age. If the calculated ratio mismatches withthe normal ratio, then the patient is detected with brain abnormality. MRI enables one to check individual slices of the brain for any AD-related abnormality within the brain. It is also possible to rotate the 3D model created with the help of MRI for analysing various parts of the brain to arrive at adiagnostic decision quickly for AD. Currently, various research works are bringing more advancements for MRI toward the detection and diagnosis of AD.

References

[1] Biju, K. S., Alfa, S. S., Lal, K., Antony, A., & Akhil, M. K. (2017). Alzheimer’s detection based on segmentation of MRI image. Procedia computer science, 115, 474-481.

[2] Xian, J. F., Chen, M., Jīn, Z.Y., Magnetic resonance imaging in clinical medicine: current status and potential future developments in China. Chin Med J (Engl). 2015 Mar 5; 128 (5):569-70.doi: 10.4103/0366-6999.151637. PMID: 25698184; PMCID: PMC4834763.

[3] Jack Jr, C. R., Bernstein, M. A., Fox, N. C., Thompson, P., Alexander, G., Harvey, D., & Weiner, M. W. (2008). The Alzheimer's disease neuroimaging initiative (ADNI): MRI methods. Journal of Magnetic Resonance Imaging: An Official Journal of the International Society for Magnetic Resonance in Medicine, 27(4), 685-691.

[4] Vemuri, P., & Jack, C. R. (2010). Role of structural MRI in Alzheimer's disease. Alzheimer's research & therapy, 2(4), 1-10.

[5] Munoz, D. G., & Feldman, H. (2000). Causes of Alzheimer's disease. Cmaj, 162(1), 65-72.

[6] Sandbrink, R., Hartmann, T., Masters, C. L., & Beyreuther, K. (1996). Genes contributing to

Alzheimer’s disease. Mol Psychiatry, 1(1), 27-40.

[7] Smith, M. A., Sayre, L. M., Monnier, V. M., & Perry, G. (1995). Radical AGEing in Alzheimer's disease. Trends in neurosciences, 18(4), 172-176.

[8] Smith, M. A., Harris, P. L. R., Sayre, L. M., Beckman, J. S., & Perry, G. (1997). Widespread peroxynitrite-mediated damage in Alzheimer’s disease. Journal of Neuroscience, 17(8), 2653-2657.

[9] Hensley, K., Carney, J. M., Mattson, M. P., Aksenova, M., Harris, M., Wu, J. F., & Butterfield, D. A. (1994). A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. Proceedings of the National Academy of Sciences, 91(8), 3270-3274.

[10] McGeer, P. L., & McGeer, E. G. (1995). The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Research Reviews,21(2), 195-218.

[11] Rapoport, S. I., Pettigrew, K. D., & Schapiro, M. B. (1991). Discordance and concordance of dementia of the Alzheimer type (DAT) in monozygotic twins indicate heritable and sporadic forms ofAlzheimer's disease. Neurology, 41(10), 1549-1549.

[12] Canadian Study of Health and Aging. (1994). The Canadian Study of Health and Aging* Risk factors for Alzheimer's disease in Canada. Neurology, 44(11), 2073-2073.

[13] Schofield, P.W., Tang, M., Marder, K., Bell, K., Dooneief, G., and Chun et al. Alzheimer’s disease after remote head injury: an incidence study. J Neurol Neurosurg Psychiatry 1997; 62:119-24.

[14] Qiu, C., Kivipelto, M., & Von Strauss, E. (2022). Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. Dialogues in clinical neuroscience.

[15] Breijyeh, Z., & Karaman, R. (2020). Comprehensive review on Alzheimer’s disease: causes and treatment. Molecules, 25(24), 5789.

[16] Berrios, G. E. (1990). Alzheimer's disease: A conceptual history. International journal of geriatric psychiatry.

[17] St George-Hyslop, P. H., & Petit, A. (2005). Molecular biology and genetics of Alzheimer's disease. Comptes rendus biologies, 328(2), 119-130.

[18] Chandra, A., Dervenoulas, G., & Politis, M. (2019). Magnetic resonance imaging in Alzheimer’s disease and mild cognitive impairment. Journal of neurology, 266(6), 1293-1302.

[19] Ballard, C., Gauthier, S., Corbett, A., Brayne, C., Aarsland, D., & Jones, E. (2011). Alzheimer's disease. the Lancet, 377(9770), 1019-1031.s.

Research Article

Assessment of groundwater quality of selected agricultural and industrial sites of Gandhinagar District, Gujarat, India

Sachin Vaidh, Bhargav Lakod, Uday Kansara, Dharni Parekh, and Gajendra Singh Vishwakarma*

Department of Biotechnology and Bioengineering, Institute of Advanced Research (IAR), Koba, Institution al Area, Gandhinagar 382426, India

**Corresponding Author: gajendra.singh@iar.ac.in*

Abstract

The suitability of the groundwater quality of 35 samples collected near Gandhinagar's agricultural and industrial sites for drinking purposes was evaluated using various water quality parameters. Standard methods as per the American Public Health Association (APHA) for physicochemical analysis of water samples were employed. The results of the analysis carried out showed the following concentration ranges: pH (7.13±0.2-8.9±0.1), TDS (115±2.3-2210±5.6 mg/l), EC (210±5.3-4200±12.8µS/cm), TH (60±5-300±11.5mg/l), Sulphate (13±5.5-190±12.3 mg/l), Fluoride (0.05±0.02-3.60±0.04mg/l), Cu (0.11±0.05-0.14±0.08 mg/l), Cr (0.001±0.05-0.02±0.04mg/l). Aside from that, heavy metals such as arsenic, lead, and mercury were analysed in the same sample, but their concentrations were below thedetection limit in majority of the samples. Overall, some of the samples collected near contaminatedsites showed concentrations of different parameters above the standards set by both national (CPCB) and international (WHO, 2011) bodies for drinking water. So, the study suggests that the groundwater in the nearby area of contaminated sites is chemically unsuitable for drinking purposes.

Keywords: groundwater quality, gandhinagar, heavy metal analysis, total dissolved solids, drinking water

Introduction

Water possesses a number of significant characteristics that make it an essential source of life - sustaining treatment for all living species on the earth. Water is renowned as the pure substance becauseof its remarkable capacity to dissolve a wide range of natural and man-made compounds. Drinking water, also known as potable water, is odourless, carbonate-free, and neither hard nor soft. If any parameter is present in excess concentration in water, it may cause sickness, and such water is referred to as unclean water and should not be consumed. Impure water causes a variety of disorders affectingthe digestive, respiratory, and circulatory systems, among other things. Most of the fraction of the drinking water comes from the ground water. Groundwater contamination is the alteration of water's physical, chemical, and biological qualities, limiting or prohibiting its usage in the different applications where it is regularly used. Human caused groundwater contamination poses unique challenges not seen in surface water pollution such as rivers, lakes, and streams (Knopek and Dabrowska,2021). Ground water quality can be influenced by household, governmental, corporate, industrial, and agricultural operations. In recent years, the demand for groundwater has deteriorated, which creates a need to monitor the quality of groundwater. In case of Gujarat, it is India’s most industrialized and urbanized state. As per the Gujarat Industrial Policy, 2020, the state accounts for

almost 8% of India's Gross Domestic Product (GDP) (GoG, 2018). As per the Annual Survey of Industries (ASI) 2017–18,Gujarat stood first in India in terms of industrial output, with ~17% of India's output due to the hub ofmanufacturing sectors such as autos and auto components, chemicals and petrochemicals, drugs and pharmaceuticals, cement, textiles, engineering, gems and jewellery, and ceramics (ASI, 2018). With the development, the state is also facing the environmental degradation, especially the ground water pollution (Modi et al., 2022; Sharma et al., 2015). Both of the industrial area as well as the agricultural sites are becoming the sink of various chemicals that are contaminating the ground water of the nearby area. As per the Central Ground Water Board (CGWB), 21 districts of Gujarat have reported issues of high EC levels, 17 districts have reported high fluoride content, 23 districts have high nitrate presencein groundwater, about 12 Gujarat districts have reported high arsenic, and 13 districts have reported high iron content (CGWB, 2018). Due to the increasing population load and spread in industrial cluster in the nearby area of Gandhinagar, there is a need of continuous monitoring of water quality parameters. From the last five years, only few studies are available of this area, therefore current study is considering the selected industrial sites and agricultural sites of Gandhinagar district for assessmentof water quality in terms of different physical and chemical parameters.

Material and methods

1. Sample collection

The groundwater samples were collected ~~on~~ between Dec- March 2020-21 from GIDC Sector25, Sector 28, Prabhupura, Sargasan, Valad, Koba, Kudasan of Gandhinagar district. The details of sampling site is given in the Fig1.

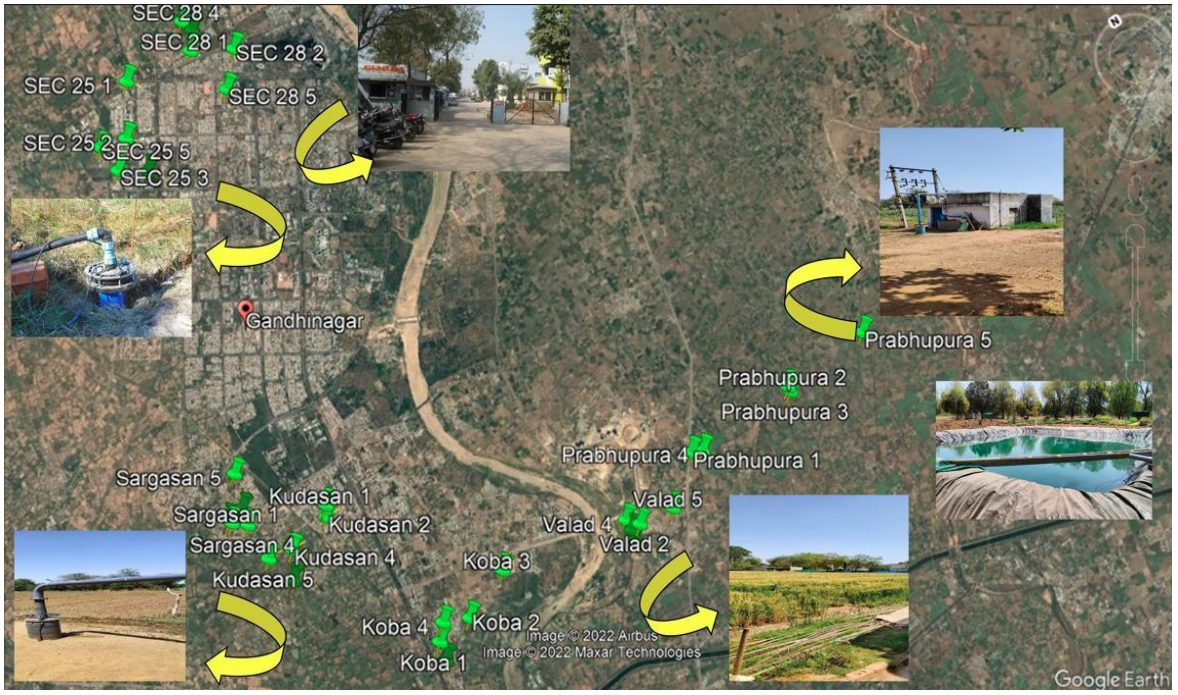


Figure 1: Location map of different sampling sites

2. Analysis of physico-chemical parameters

Temperature, pH, electrical conductivity, total dissolved solid (TDS) and dissolved oxygen (DO) of water

samples were determined by multi-parameter instrument of Hanna (HI98130).

3. Heavy metal analysis

Heavy metals (As, Cd, Hg, Pb, Cr and Ni) analysis of ground water sample were conducted with the help of Inductive Coupled Plasma Mass Spectrometry (ICPMS: Thermo Scientific Model (iCAPQc) atCentral University of Punjab, Bathinda, India.

4. Total hardness

Total hardness is due to the presence of bicarbonates, chlorides, and sulphates of calcium and magnesium ions. The total hardness of water is estimated by titrating the water sample against EDTA using Eriochrome Black-T (EBT) indicator (APHA, 1999).

5. Sulfate

It is analysed by turbidimetric method as per standardized by APHA, 1999. In this method,sulfate ion is converted to a barium sulfate suspension under controlled conditions. The resulting turbidity was determined by spectrophotometer at 420nm and compared with a curve prepared from standard sulfate solution (APHA, 1999).

6. Copper

The copper ions are determined by the colorimetric/spectroscopic method as per APHA protocols. In this method, Cuprous ion forms a water-soluble orange-colored chelate with bathocuproine disulfonate (2, 9-dimethyl-4, 7-diphenyl-1, 10-phenanthrolinedisulfonic acid, disodium salt). In this method, the sample was buffered at a pH of about 4.3 and reduced with hydroxylamine hydrochloride. The absorbance was measured at 484 nm (APHA, 1999).

Results and discussion

Physico-chemical parameters

The pH of the samples were observed in the range of 7.13±0.2-8.9±0.1 (Fig 2). The pH of the agricultural sites of Prabhupura and Valad area was near 7, while the pH of the industrial area was slightly basic i.e., between 7.9 - 8.9. The alkaline nature of the ground water near the industrial area may be due to presence of salts in the water or deposition of different chemicals in the nearby area. In the case of TDS, majority of the samples showed more than 500 ppm, which is higher than the permissible limit of drinking water quality. Again the samples of industrial sites showed TDS higher than 1200. The sample no. SEC28 3 which belonged to GIDC area showed the maximum TDS 2200 (Fig 3). Similarly the electric conductance of the EC of the samples was also observed higher in sample no. SEC28 3.

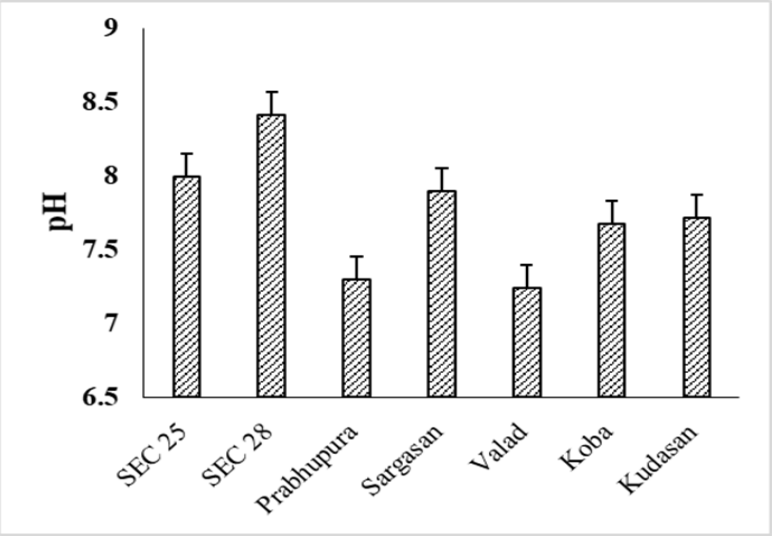


Figure 2: pH of the ground water collected from different area of Gandhinagar

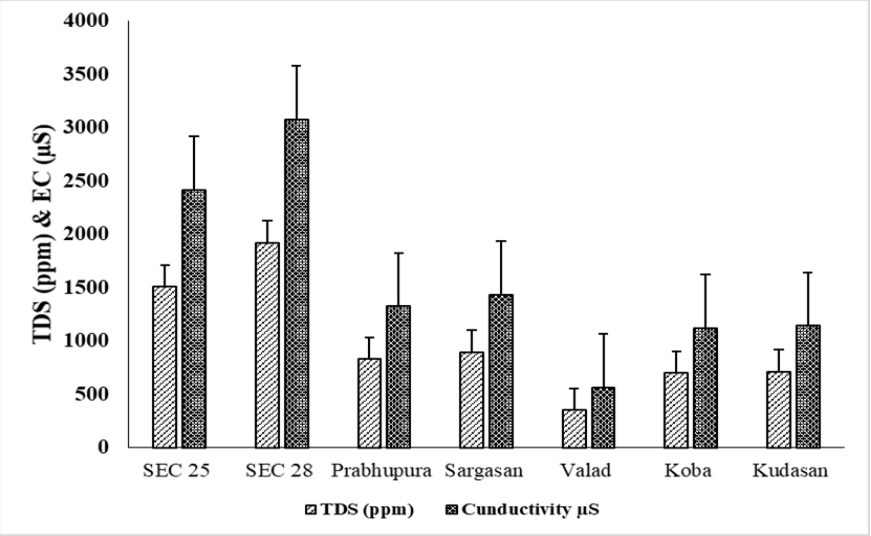


Figure 3: TDS and EC of the ground water collected from different area of Gandhinagar

The study showed that the hardness of the ground water was 60±5-300±11.5mg/l. The highest value was reported in sample collected from the sec 28 (Fig 4). The high value indicates the influence of discharged salts and soluble solids from industrial waste or effluents (Wdowczyk and Szymańska-Pulikowska, 2018). Further, the sulphate concentration of the ground water was found in range between 13±5.5- 190±12.3 ppm. The lowest value 13 ppm was reported in the valad site, while the highest value 190 ppm was reported in the sec 28 area (Fig 4). The higher range of sulphate indicates the percolation of industrial discharge in the aquifers of groundwater of the surrounding area (Przydatek and Kanownik, 2021).

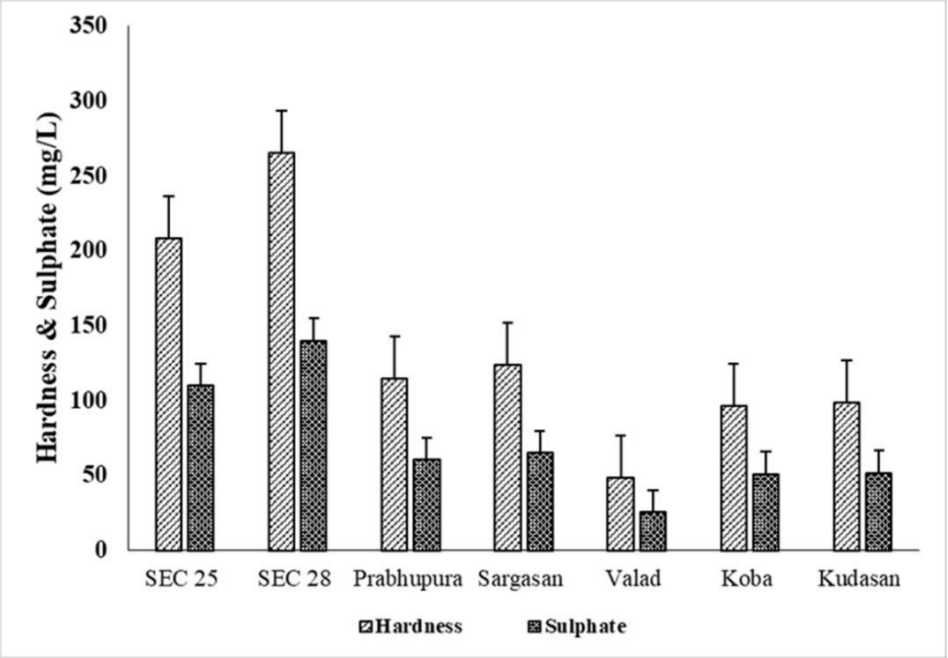


Figure 4: Hardness and sulphate concentration in the ground water collected from different areas of Gandhinagar

As per the International Standard (IS), permissible limit of fluoride for drinking is 1-1.5 mg/l.The fluoride value in some samples of ground water sample of SEC 28 and 25 was exceeding the permissible limit. While, in other areas, no sample was found with higher value than permissible limit.Similarly, in case of Copper the IS, permissible limit of copper for drinking is 0.05-5.0 mg/l. The copper value in most of the samples was not exceeding the value of permissible limit. In case of Cr the IS, the acceptable limit for drinking is 0.05 mg/l. The chromium value of water sample of all the sites was 0.001±0.05-0.02±0.04mg/l. In this result, no sample was found with higher value than permissible limit (Fig. 5).

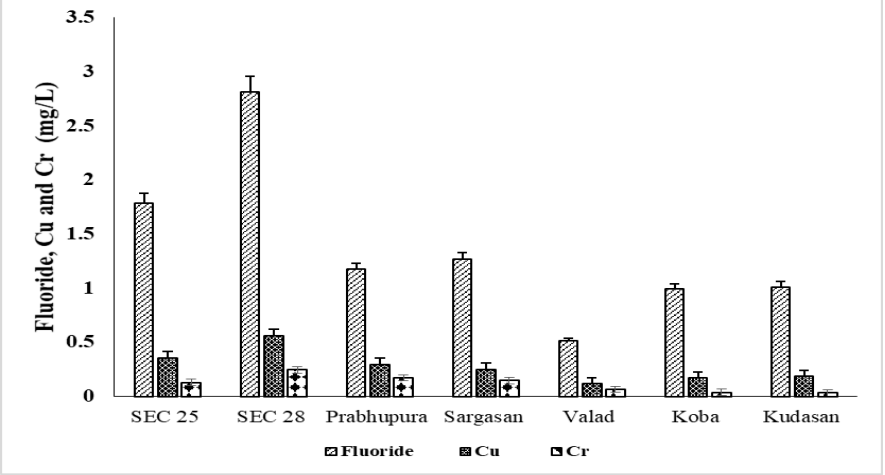


Figure 5: Fluoride, Cu and Cr concentration in the ground water collected from different areas of Gandhinagar

The heavy metal concertation in arsenic, lead, and mercury were analysed in the same samples but their concentrations were below the detection limit in majority of the samples.

Conclusion

Concentration of various physico-chemical parameters including (pH, TDS, conductivity, hardness, sulphate, F, Cr, Cu) in some of the samples collected from the outer periphery of industrial area was very high. While, the concentration of same physico-chemical parameters collected from agricultural sites were moderately high. The high values of the physico-chemical parameters of the groundwater of nearby sites of industrial area is may be due the leaching or percolation salts and pollutants in the ground water sources. Therefore, the study suggested that the groundwater in the nearby area of contaminated sites is chemically unsuitable for drinking purposes without any type of purification process.

Acknowledgement

I would like to acknowledge IAR and Gujarat State Biotech Mission (Grant no. GSBTM L1Y5SU) for their support in this work.

References

APHA. (1999). Standard Methods; Standard Methods. https://www.standardmethods.org/action/showCitFormats?doi=10.2105%2FSMWW.2882.054 ASI. (2018). Annual Survey of Industries. http://microdata.gov.in/nada43/index.php/catalog/ASI

CGWB, R. (2018). *Central Groundwater Board, Annual Report 2018–18(Central Ground Water Board, Ministry of Water Resources, Government of India)*. http://cgwb.gov.in/Annual-Reports/Final_Annual_Report_CGWB_2016-17_%2003.01.2019.pdf.

GoG. (2018). *Gujarat Industrial Policy*. https://ic.gujarat.gov.in/industrial-policy.aspx.

Knopek, T., & Dabrowska, D. (2021). The Use of the Contamination Index and the LWPI Index to Assess the Quality of Groundwater in the Area of a Municipal Waste Landfill. *Toxics*, *9*(3), Article 3. https://doi.org/10.3390/toxics9030066

Modi, A., Khatri, N., Tyagi, S., & Jha, A. K. (2022). Analysis of Contaminants in Sewage and Groundwater: Future Impact on Human and Animal Health. *Environmental Claims Journal*, *34*(3), 208–221. https://doi.org/10.1080/10406026.2021.1907992

Przydatek, G., & Kanownik, W. (2021). Physicochemical indicators of the influence of a lined municipal landfill on groundwater quality: A case study from Poland. *Environmental Earth Sciences*,*80*(13), 1–14.

Sharma, M. K., Jain, C. K., Rao, G. T., & Rao, V. V. S. G. (2015). Modelling of lindane transport in groundwater of metropolitan city Vadodara, Gujarat, India. *Environmental Monitoring and Assessment*, *187*(5), 295. https://doi.org/10.1007/s10661-015-4522-6

Wdowczyk, A., & Szymańska-Pulikowska, A. (2018). Analysis of possibilities of water environment assessment in the area of the landfillment of municipal waste. *Ecological Engineering & Environmental Technology*, *19*(6), 57–64.

NATURAL SCIENCES

The Journal of the Institute of Advanced Research

Volume 3; Issue 1 (2022)

The University for Innovation

Institute of Advanced Research
Koba Institutional Area
Gandhinagar–382426,
Gujarat, India