UPDATES IN INTERNAL SCIENCES FOR 2021

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Ramazan GİDEN & Şerif KURTULUŞ

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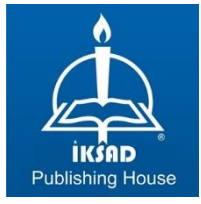

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PREFACE

The solution of today's health problems will be realized thanks to science in the future as it has been in the past. The social benefits of scientific research carried out by scientists in the field of health by making sacrifices and joining their day and night are indisputably high. In this context, we tried to collect the studies of health professionals in this book called "Updates in Internal Sciences in 2021". Hope it is useful.

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CHAPTER 1

COVID-19 and GENOME STRUCTURE

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INTRODUCTION

Coronaviruses (CoVs) are cause some diseases both animals and humans. Human coronaviruses (HCoVs), which cause acute upper respiratory tract infections, were first identified in 1962. HCoVs were frequently related with serious upper and lower respiratory tract infections. Over the last two decades, two pathogenic human coronaviruses have been identified, including severe acute respiratory syndrome (SARS-CoV) and the Middle East respiratory syndrome (MERS-CoV), which cause severe respiratory infections (Habas et al., 2020).

SARS-CoV-2, a new coronavirus (New Coronavirus Disease (COVID-19)), has spread rapidly like a pandemic since it emerged in Wuhan, China in 2019 (Hosseini et al., 2020). It has become a serious global public health issue with the rapid spread of SARS-CoV-2, a common clinical symptom of pneumonia. The World Health Organization (WHO) reported a new coronavirus as a result of whole-genome sequencing. As a result of the researches, WHO confirmed that the new Coronavirus was responsible for these clinical symptoms and declared the disease as COVID-19 (Liu et al., 2020; Samudralavn et al., 2020). The genomic sequence of the new virus has been identified as the SARS-CoV-2 virus by the International Committee on Virus Taxonomy (ICTV), as it is associated to SARS-CoV (Liu et al., 2020).

In the last two decades, SARS-CoV and MERS-CoV have caused epidemics with mortality rates of nearly 9.5% and 34.4%,

respectively. COVID-19, on the other hands, was the third-highest disease becomed with a lower death rate after SARS and MERS, although it differ country. (Mohamadian et al,. 2021).

It is recognized as the testof patients' bronchoalveolar lavage fluids by next-generation-sequencing (NGS), determined as a novel viral RNA genome unlike with previous viruses. Further studies determined that this new virus is a member of beta coronaviruses. Some phylogenetic studies showed that the beta-coronavirus genus of the virus is a new zoonotic virus belonging to the Sarbecovirus subgenus. To date, seven coronavirus members have been identified and reported to infect humans. Other members of the coronaviruses that infect humans were OC43, 229E, NLG3, and HKU1 and can cause minor respiratory infection symptoms in immunocompetent individuals. SARS-CoV-2 is the seventh member of the coronavirus family, which causes symptoms associated with pneumonia, similar to the SARS and MERS viruses. As a result of whole-genome analysis studies, it was shown that the SARS-CoV-2 genome is 96% some to a bat-derived coronavirus, and it was reported that the bat could be the source for COVID-19 (Esmaeilzadeh and Elahi 2020).

CoVs are viruses with a positive single-stranded RNA genome and pathogenic envelope. SARS-CoV-2 is a more pathogenic virus compared to SARS-CoV and MERS-CoV. To understand its pathogenesis mechanism, virulence and to advance potent therapeutic strategies, SARS-CoV-2 should be examined comprehensively. CoVs

belong to the Coronaviridae family of the order Nidovirales (Nagvi et al., 2020).

In general, there are four genera within the Coronaviridae family: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus (α, β, γ and δ). (Wang et al., 2020). Among these coranaviruses, Alphacoronavirus and Betacoronavirus infect mammals, Gammacoronavirus bird species, and Deltacoronavirus both mammals and prey. Human coronavirus OC43 has been named β-coronaviruses, including mouse hepatitis coronavirus (MHV), bovine coronavirus (BCoV), bat coronavirus HKU4, SARS-CoV, MERS-CoV, and SARS-CoV-2. SARS-CoV, MERS-CoV and SARS-CoV-2 are zoonotically transmitted and spread between humans in close contact. (Nagvi et al., 2020).

Coronaviruses have the largest genomes (26.4–31.7 kb) of all known RNA viruses (Mousavizadeha and Ghasemi 2021). The size of the genome increases genomic plasticity. Thus, it allows modification through mutations and recombination. This allows for higher genetic diversity and a higher chance of cross-species transmission (Habas et al., 2020). G + C contents in their genomes vary between 32-43%. A variable number of small open reading frames (ORFs) exist between the some preserved genes (ORF1ab, spike, envelope, membrane, and nucleocapsid e.g.) and, downstream of the nucleocapsid gene in coronavirus strains. The viral genome contains specific features, including a unique N-terminal fragment within the Spike protein. The genes for the major structural proteins in all coronaviruses occur in the

5'–3' order as Sipeke (S), Envelope (E), Matrix protein (M), and nucleoprotein (N) (Mousavizadeha and Ghasemi 2021).

SARS-CoV-2 are positive single-stranded, spherical or pleomorphic enveloped particles associated with N protein in a capsid composed of M protein. The envelope contains S protein structures. Some coronaviruses also contain hemeagglutinin-esterase protein (HE) (Mousavizadeha and Ghasemi 2021).

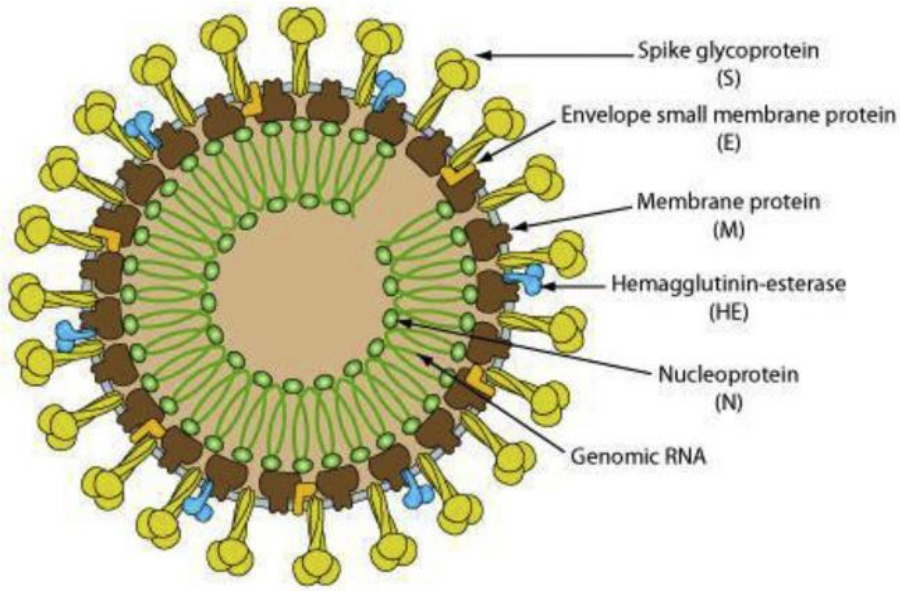

Figure 1. Structure of Coronavirus (Mousavizadeha and Ghasemi 2021).

The entry of CoVs into the host cell is provided by S proteins that attach to host cell receptors. The S protein binds to the host receptor with the receptor-binding domain in the S1 subunit. Fusion to the cell membrane takes place with the S2 subunit.

In the researches, the genome size of SARS-CoV-2 was determined to be ~29.9 Kb in the NCBI genome database (NC_045512.2). It was observed that the studied SARS-CoV-2 consisted of 13-15 (12 functional) ORFs containing ~30,000 nucleotides in its genetic structure. The genome contains 38% of the GC content and 12 expressed proteins and 11 protein-coding genes. The genetic arrangement of ORFs is similar to SARS/ MERS-CoV. ORFs are composed as replicase and protease (1a-1b) and S, E, M, and N proteins following a typical 5'-3' order of view, and this portion is considered to be major drug or vaccine targets. They also play significant roles in the fusion and survival of the virus into host cells. The entire genome of SARS-CoV-2 encodes a polyprotein of approximately 7096 residues long, consisting of structural (S, E, M, and N) and 16 nonstructural proteins (NSPs).

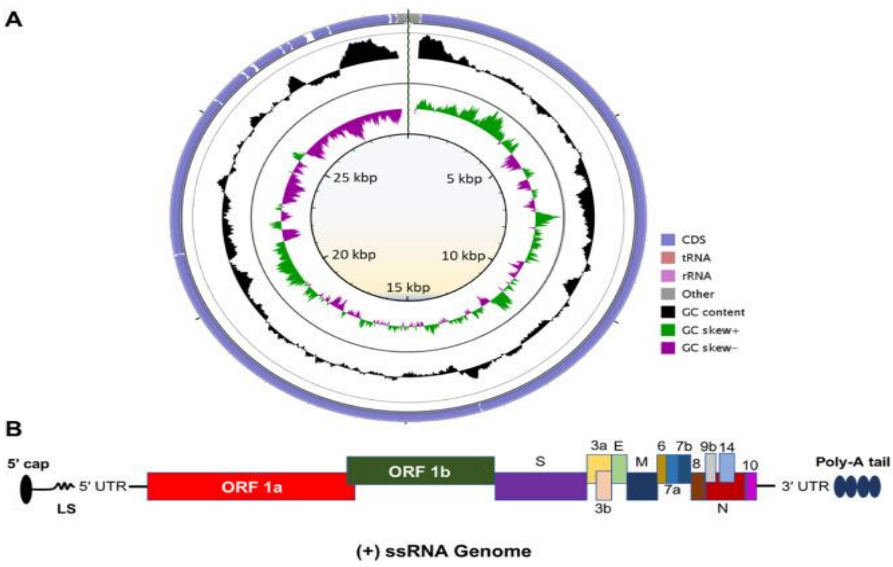

Figure 2. Genome structure of SARS-CoV-2 (Nagvi et al., 2020).

NSPs have various functions in biological events (replication, translation, immune response blocking, and RNA stabilization) (Raskin 2021).

Nsp1 regulates RNA replication and processing. Nsp2 regulates the host cell survival signaling pathway. Nsp3 functions to cleave the polyprotein into its different proteins. Nsp4 anchors the viral replication-transcriptional complex to modified endoplasmic reticulum (ER) membranes. Nsp5 joins in the viral polyprotein process during replication. Nsp6 is involved in the initial induction of autophagosomes from the host ER. Nsp7 is an RNA-dependent RNA polymerase. Nsp8 forms a hexadeca-meric supercomplex that adopts a hollow cylinder-like structure containing duplication with Nsp7. Nsp9 acts as an ssRNA binding protein. Nsp10 has a significant role in cap methylation of viral-mRNAs. Nsp11 consists of 13 amino acids and their function are not yet known. Nsp12 is responsible for the replication and transcription of the Covs RNA genome. Nsp13 participates in the zinc-binding, replication and transcription process. Nsp14 has exoribonuclease activity and N7-guanine methyltransferase, Nsp15 has Mn(2+)-dependent endoribonuclease activity and Nsp16 is a 2'-O-ribose methyltransferase (Wang et al., 2020; Nagvi et al., 2020).

The S protein is a major, multifunctional viral transmembrane protein. It is contained in a trimer on the virus surface, giving the virus a corona or crown-like appearance. It interacts with the host cell receptors to allow the entry of infectious virus particles into the cell.

The M protein, which is a viral protein and abundant in the virus particle, gives shape to the viral envelope. This protein also binds to the nucleocapsid and plays role as the central regulator of the coronavirus. E protein is small structural proteins, is involved in the pathogenesis, aggregation and release of the virus. N protein, on the other hand, has been observed in studies to acts in the formation of complexes with the viral genome and facilitate its interplay when needed during virion formation and increase the transcription efficiency of the virus (Dhama et al., 2020).

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CHAPTER 2

THE S GENE PRIMER DESIGN FOR THE DETECTION OF SARS-COV-2 VIRUS

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INTRODUCTION

1. SARS-CoV-2 & COVID-19

COVID-19 is a disease caused by the SARS-CoV-2 virus which was first identified in December 2019 in Wuhan, China (Parikesit & Nurdiansyah, 2020). Since then, the virus has infected 191,725,149 people and claimed 4,112,929 lives as per 27th of August 2021 (Worldometer, 2021). COVID-19 spreads rapidly throughout the world. Therefore, this disease is declared a global pandemic by the World Health Organization (WHO) (Pusparini, 2020) so that COVID-19 becomes a public health threat because it is a new virus that is not recognized by the immune system, and the virus spreads vastly (Cascella et al., 2021). Unfortunately, no effective drug has been found to cure patients and make them free from the COVID-19, but scientists have been studying the virus thoroughly to develop the vaccine that will effectively protect people from COVID-19. However, many vaccines cannot ensure 100% protection to the people due to the constant mutation of the virus (Kirchdoerfer & Ward, 2019). In other words, vaccines play an essential role in producing antibodies to protect the body, not antivirus, that makes people immune to COVID-19 (Darby & Hiscox, 2021).

As a RNA virus, SARS-CoV-2 continually mutates over time, as do all viruses. The majority of the modifications have little or no effect on the virus's characteristics. However, mutations can affect the nature of the virus, such as how easily it spreads, the severity of the disease it causes, or the effectiveness of vaccines, therapeutic medications,

diagnostic instruments, and other public health and social measures (World Health Organization, 2021). Until now, scientists have identified 12 new variants of SARS-CoV-2 that are a result of the mutation, including alpha variant (B.1.1.7) from England, beta variant (B.1.3.5.1) from South Africa, gamma variant (P1) from Brazil, and delta variant (B.1.6.1.7.2) from India, which was considered as the variant of concern by WHO (World Health Organization) (Cascella et al., 2021). The new strains of the SARS-CoV-2 virus that were considered the variant of concern were informed that the viruses spread faster than the original strains. Nowadays, the SARS-CoV-2 virus with delta variant (B1617.2), which was first found in India, has spread rapidly worldwide (World Health Organization, 2021).

The WHO contemplated the names of Greek gods and goddesses for a while but ultimately decided against them. The option of simply numbering them one, two, three, and so on was examined but dismissed since it was feared that this would confuse the names given to the viruses in genetic sequence databases that follow the evolution of the SARS-CoV-2 virus. WHO brought together experts to come up with a naming scheme, some of whom were members of the International Committee on Taxonomy of Viruses (ICTV). They approved the Greek alphabet naming idea. As a result, the variants of the SARS-CoV-2 virus that causes COVID-19 was named by ICTV that oversees naming novel virus species (Branswell, 2021). The reason why WHO made a naming scheme for SARS-CoV-2 variants using the Greek alphabet are to make it easier to discuss, make it

easier for researchers to discuss, make it easier to remember, and eliminate some of the stigmas that make countries of origin feel cornered for the first time a variant appears. Currently selected is the order of the Greek alphabet which contains 24 letters, but don't know what to do if they run out. Epsilon, Zeta, Eta, Theta, and Iota have been used as the names of the new variants of the coronavirus (World Health Organization, 2021).

COVID-19, which is caused by the SARS-CoV-2 virus originating from bats, this virus is transmitted through droplets, especially when the infected cough, sneeze, or talk, small droplets come out from the nose and the mouth along with the virus (Yiwei, 2020). The Incubation period of SARS-CoV-2 is between 5 until 14 days (Yang, Xiao, & Ye, 2020).

During this pandemic, the elderly and vulnerable populations need to be protected from the spread of COVID-19. Most individuals infected with the SARS-COV-2 are asymptomatic or have only mild to moderate symptoms, with flu-like symptoms or other flu infections, so that if we lose the ability to follow in the footsteps of everyone who has ever been If infected with SARS-CoV-2, then the process of identifying potentially infected individuals will be difficult (Pusparini, 2020).

2. Comparison between SARS-CoV, MERS-CoV, and SARS-CoV-2

SARS-CoV and MERS-CoV are the member of the coronaviridae family and have many similarities and differences. SARS-CoV is a severe acute respiratory syndrome that is caused by a coronavirus and infects humans, bats, and civet cats. This virus was first discovered in China in 2002 and was identified in 2003, and eventually spread to North America, South America, Europe, and Asia. During the SARS pandemic, more than 770 deaths were reported from this disease. SARS-CoV attacked the upper respiratory system (Giannis, Ziogas, & Gianni, 2020), whereas MERS (Middle East Respiratory Syndrome coronavirus or MERS-CoV) is a viral infectious disease that attacks the respiratory system. This virus is also a zoonotic virus which means it is transmitted from animals to humans. The origin of the virus is not fully known. Research from the Annals of Saudi Medicine stated that at first, humans were suspected of contracting the MERS-CoV virus from camels through direct or indirect contact. This virus is found in the body of one-humped camels in several countries in the Middle East, Africa, and South Asia. There have been 2,494 cases of MERS reported worldwide, with 858 deaths. According to WHO, MERS infection occurs mainly from close person-to-person contact. Although the number of spread and the death toll is smaller, the death rate from MERS is very high, which is 34.45% (De Groot et al., 2013). Many of the COVID-19 medical breakthrough discoveries were made based upon the previous research of MERS-CoV and

SARS-CoV-2. It also serves as the starting point of the PCR protocol development to detect SARS-CoV-2 virus.

3. PCR as the kit to detect the presence of SARS-CoV-2 virus

As for the responses, governments in every country need to track the population by implementing the COVID-19 test to detect the presence of the COVID-19 virus. WHO recommends the Reverse Transcription Polymerase Chain Reaction (RT-PCR) method as the gold standard for the diagnosis of SARS-CoV-2 infection (World Health Organization, 2020b). The RT-PCR method is used to detect the presence of the virus, which targets the SARS-CoV-2 genome with the primer and probe (Agustina & Fajrunni'mah, 2020).

4. PCR conventional & RT-PCR

There are the difference between PCR conventional and RT-PCR from many aspects that explained in the table 1.

Aspects	PCR conventional	RT-PCR
Enzyme	PCR requires Taq Polymerase (Mo et al., 2012)	RT-PCR requires Enzyme Reverse Transcriptase & DNA polymerase (Fisher, 2020)
Reagent	PCR requires DNA template, two primers, Taq polymerase or another DNA polymerase, buffer solution, dNTPs, and divalent cations such as Mg2+ (Mo et al., 2012)	RT-PCR requires Template cDNA, Upstream and downstream primers, DNA Polymerase with 5× reaction buffer, dNTP mixture, Molecular grade and nuclease-free water, Agarose, Ethidium bromide solution, and 50× TAE (Mo et al., 2012)
Usage	Measures the amount of PCR product that has accumulated at the end of	Measures the rate of PCR amplification in real time.

	the PCR cycle.	
Result	Semi-Quantitative (by comparing the strength of the amplified band on a gel to known concentration standards)	Results in Quantitative (data is taken during the exponential growth (log) phase of PCR, when the PCR product quantity is proportional to the amount of template nucleic acid)
Application	Amplification of DNA for Sequencing, Genotyping, and Cloning	Quantitation of gene expression, Microarray verification, Quality control and assay validation, Pathogen detection, SNP genotyping, Copy number variation, MicroRNA Analysis, Viral quantitation, and siRNA/RNAi experiments
Measurement	At the plateau phase of the graph, which gives variable results	The exponential phase, which gives more accurate results

Table 1. The difference between PCR conventional and RT-PCR (Zhao et al, 2007).

5. PCR & antigen swab test

Basically, the PCR swab test and antigen swab test sampling methods are similar. Only the molecular mechanism and the biochemistry basis are different. PCR swab test detects the genetic material of the SARS-CoV-2 coronavirus, which causes COVID-19. The PCR swab test begins with taking a sample of respiratory fluid or mucus from the nose and throat with a long cotton swab-like device, the process takes 2-3 hours or even more (Agustina & Fajrunni'mah, 2020). Whereas the antigen swab tests detect the specific protein of the SARS-CoV-2 virus. An antigen is a substance that can stimulate immunity. These substances can be proteins, polysaccharides, and others. When infected with a virus, the body will naturally respond by secreting certain specific proteins. For example, the virus that causes COVID-

19 has several recognized antigens, such as nucleocapsid phosphoproteins and spike glycoproteins. An antigen swab test can see the presence of antigen in the body, so it can be known whether a person is infected with the coronavirus or not. PCR swab test detects the most accurate COVID-19 test. The accuracy can reach 80-90%. Meanwhile, the antigen swab test has an accuracy level below the PCR swab test (Makarim, 2021). A positive result means that there are some viruses in the sample, whereas a negative result means that there is no virus in the sample (Pusparini, 2020). Also, a negative COVID-19 test result (both from the PCR swab test and antigen swab test) are not a guarantee that are uninfected because the test result was negative last week, it doesn't mean the test result is negative now (World Health Organization, 2021).

Ct (Cycle of threshold) value is the number of cycles generated in searching for viral genetic material from mucus samples results of COVID-19 patients (Rumah Sakit Universitas Indonesia, 2021). Ct indicates how many amplification cycles for the virus signal to be detected. If the Ct value is low, it means that the genetic virus is quickly detected. The normal Ct value is >25 (for antigen swab) and according to Rumah Sakit Universitas Indonesia (2021), the normal Ct value of RT-PCR is >34.

6. PCR Primer

Successful designing primers for PCR is highly dependent on the used primers. The primer functions as a barrier for the target DNA fragment to be amplified and provide the hydroxy group (-OH) at the

3' end which is used for the existence of DNA. Primer design can be done based on the known DNA sequence or the target protein sequence. If the DNA sequence or the target protein sequence is unknown, the primer design can be based on the results of homology analysis of the DNA or protein sequences that have the closest kinship (Utomo, Ichsan, & Putri, 2019).

7. Spike glycoprotein gene

In this study, the spike glycoprotein (S) gene will be used as the target of the primer. Spike Glycoprotein is the protein that plays an important role in binding to receptors on host cells and spreading SARS-CoV-2. Therefore, this protein is a significant target for several antiviral treatments, as well as a prospective antigen for developing vaccines against SARS-CoV-2 (Ansori, Kharisma, Muttaqin, Antonius, & Parikesit, 2020). This is due to its linkage with tropism, binding to cell-surface receptors, fusion, and virus entrance into cells, which enables the virus to neutralize antibodies associated with protective immunity (Timurkan et al., 2020). Spike glycoprotein gene (S) was used as a target of the primer design of PCR because it has lower percentage of similarity (<75%) and chosen because has a lowest percentage of similarity compared with the other variants of SARS-CoV-2 so that spike glycoprotein gene become the good ideal target as the function of screening and confirmation in SARS-CoV-2 detection (Lu et al., 2020).

8. Goal of this study

This study will be done by *in silico* method that focuses on making the suitable primer for the delta variant (spike protein) of the SARS-CoV-2 virus and analyzing the combination of primer pairs used in the PCR process to create a suitable primer design for the delta variant of the SARS-CoV-2 virus.

METHODOLOGY

The pipeline could be summarized as seen in the Figure 1 and explained in the main text.

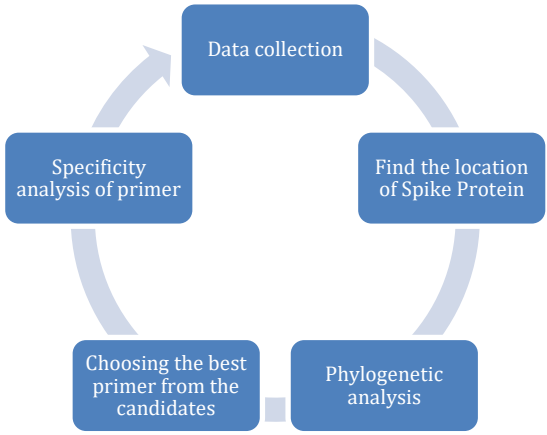


Figure 1. The brief summary of the analysis of the primer. The pipeline was inspired from existing method (Tambunan et al.,2010).

- Data collection. The samples were retrieved from NCBI virus (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/) in B.1.6.1.7.2 pangolins that range between July to August 2021 in India (MZ724415.1), Pakistan (MZ676662.1), Uzbekistan (MZ573297.1), and USA (MZ734503.1).

- Finding the location of spike glycoprotein gene (spike protein). The samples retrieved from NCBI virus are the whole genome. Deploying Geneious Prime to show the region of spike glycoprotein gene in each sample.
- Multiple Sequence Alignment (MSA) and phylogenetic tree construction analysis. Multiple Sequence Alignment (MSA) used to show the difference of the sequences, while phylogenetic tree construction used to check the relationship of the sequences, and prove and confirm the existence of a delta variant. In this process, MUSCLE alignment in the Geneious Prime and MEGA-X software will be applied to align the sequences that used to check whether the sequences have mutation or not, while the maximum-likelihood method in MEGA-X will be applied to construct the phylogenetic tree. Bootstrap values of 1000 replicates were calculated to evaluate the reliability of the phylogenetic tree (Chan et al., 2020).
- Choosing the best primer from the candidates. In this process, Primer3 (https://primer3.ut.ee) will be used to analyze the primer and choose the best primer from the candidates. In this section, researchers focus on the specificity of the primer. The best primer can recognize the target of the gene that is wanted, so that the primer specificity should be considered. Besides that, the other criteria must be considered, such as the length of the sequences, difference of Tm, %GC content, GC clamp, self-complementarity, 3' self-complementarity, runs, and repeats the presence of dimer, hairpin, and secondary structure. By using

Primer-BLAST software, the sequences of the primer candidates will be compared with the other sequence datasets from the many variants of organisms that are available in NCBI (Saraswati, Sepriyanto, & Wahyuni, 2019).

- Dimer analysis of primer. Dimer structure in the primer significantly affects the PCR primer performance. The dimer is an identification of one primer with another primer. Dimers are formed because designed primers can recognize sequences from themselves to bind to each other to form a structure. (Saraswati, Sepriyanto, & Wahyuni, 2019). To check the presence of dimer structure, researchers need to analyze the prediction of dimer structure (self dimer and cross dimer) on the candidates of primer using NetPrimer software (www.premierbiosoft.com/netprimer/).
- Hairpin analysis of primer Using NetPrimer software (www.premierbiosoft.com/netprimer/) to check and ensure that there is no hairpin on the designed primer, so that the primer can stick to the template well (Kibbe, 2007). The presence of hairpin can interrupt the stick process in the template during PCR processing. Hence, the form of hairpin in the primer should be avoided (Utomo, Ichsan, & Putri, 2019).
- Specificity analysis of primer. Using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) to validate the primer candidates that is used to ensure the primer candidates are working or not, this step can be done after

check the hairpin analysis in the primers. The criteria that the primers will work or not are that the primer can recognize the target organisms and their genes. If the primer can recognize organisms other than SARS-CoV-2 virus with high specificity, that primer will be discarded because it reduces the DNA concentration of our target (Saraswati, Sepriyanto, & Wahyuni, 2019).

RESULT & DISCUSSION

1. Samples Data Retrieval

Table 2. SARS-CoV-2 with pangolins B1617.2 from Spike Glycoprotein samples retrieved from NCBI virus that were used for study were shown (Brister et al, 2015).

Accession ID	Sample Name	S Protein Location	Release Date
MZ724415	SARS-CoV-2/human/IND/Hospital_1_28/2021	21510-25325	09-AUG-2021
MZ676662	SARS-CoV-2/human/PAK/JRCGR_SCV-103/2021	21556-25371	02-AUG-2021
MZ573297	SARS-CoV-2/human/UZB/UZB-331/2021	21531-25346	19-JUL-2021
MZ734503	SARS-CoV-2/human/USA/NMDOH-2021135848/2021	21509-25324	10-AUG-2021

2. Phylogenetic tree construction

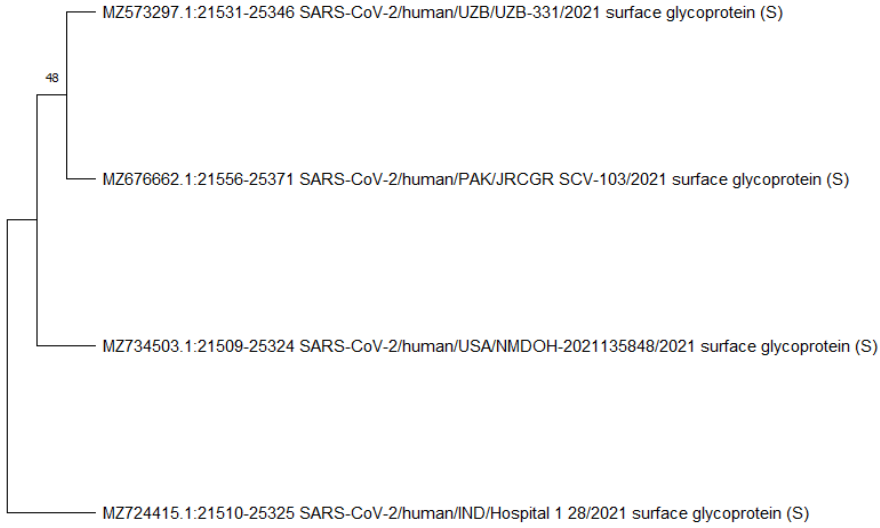


Figure 2. The phylogeny tree of the samples using Maximum-Likelihood algorithm with Bootstrap values of 1000 replicates (Hall, 2013).

The phylogeny tree of the samples is constructed using maximum-likelihood algorithm with bootstrap values of 1000 replicates in the MEGA-X software. Table 2 shows the gene samples, and the figure 2 shows that the sample from Uzbekistan and Pakistan has a near kinship because they have a similar sequence, and the USA sample is still near with a sample from Uzbekistan and Pakistan. Whereas, the sample from India as the outgroup of the phylogeny tree because it has a significant difference of the sequence. For the sample from the USA, it has the R nucleotide base in the 425th loci (figure 4a) which causes the sequence to have slight differences with others.

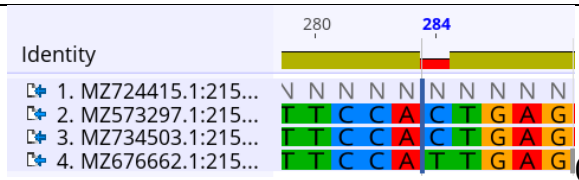
3. Mutation position of the base nucleotide

Table 3a. List of location on sequences which have a mutation. The polymorphism was tabulated based upon existing pipeline (Ray et al, 2021).

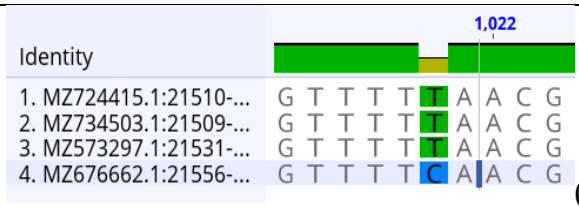
Sequence	Nucleotide base location	Nucleotide base (normal)	Nucleotide base (mutation)
MZ676662 (Pakistan)	284	C	T
MZ676662 (Pakistan)	1020	T	C
MZ676662 (Pakistan)	1495	A	T
MZ676662 (Pakistan)	2036	G	A
MZ676662 (Pakistan)	2542	A	C
MZ573297 (Uzbekistan)	3183	G	T

Table 3b. List of location on sequences which have a mutation. The polymorphism was tabulated based upon existing pipeline (Ray et al, 2021).

Sequence	Nucleotide base location	Nucleotide
MZ724415 (India)	155-428	N
MZ734503 (USA)	425	R
MZ676662 (Pakistan)	467-473	N
MZ724415 (India)	779-968	N
MZ724415 (India)	1321-1547	N
MZ724415 (India)	1561-1562	N



(a)



(b)

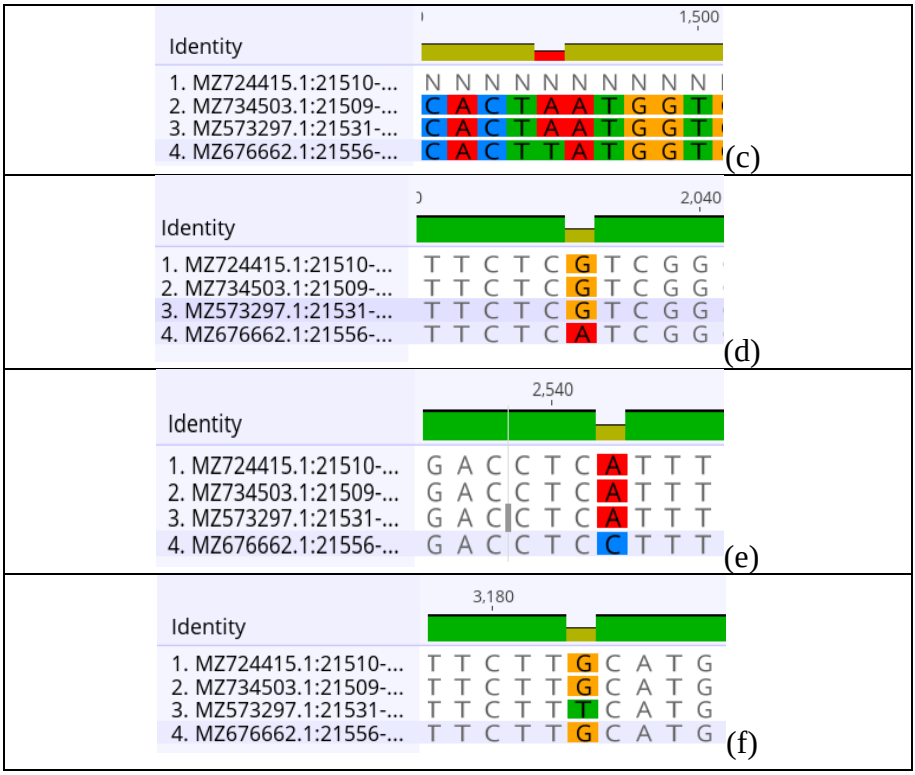


Figure 3. (a) The location of mutation of the sequence in the 284th nucleotide base location, base nucleotide that has mutation from C to T is sequence from Pakistan. (b) The location of mutation of the sequence in the 1022nd location, base nucleotide that undergoes mutation from T to C is sequence from Pakistan. (c) The base nucleotide that undergoes mutation from A to T is sequence from Pakistan in the 1495th nucleotide base location. (d) The base nucleotide that undergoes mutation from G to A is sequence from Pakistan in the 2036th nucleotide base location. (e) The base nucleotide that undergoes mutation from A to C is sequence from Pakistan in the 2542nd location. (f) The base nucleotide that undergoes mutation from G to T is sequence from Uzbekistan in the 3183th nucleotide base location. Sequence alignment visualization was depicted based on the existing method (Kearse et al, 2012).

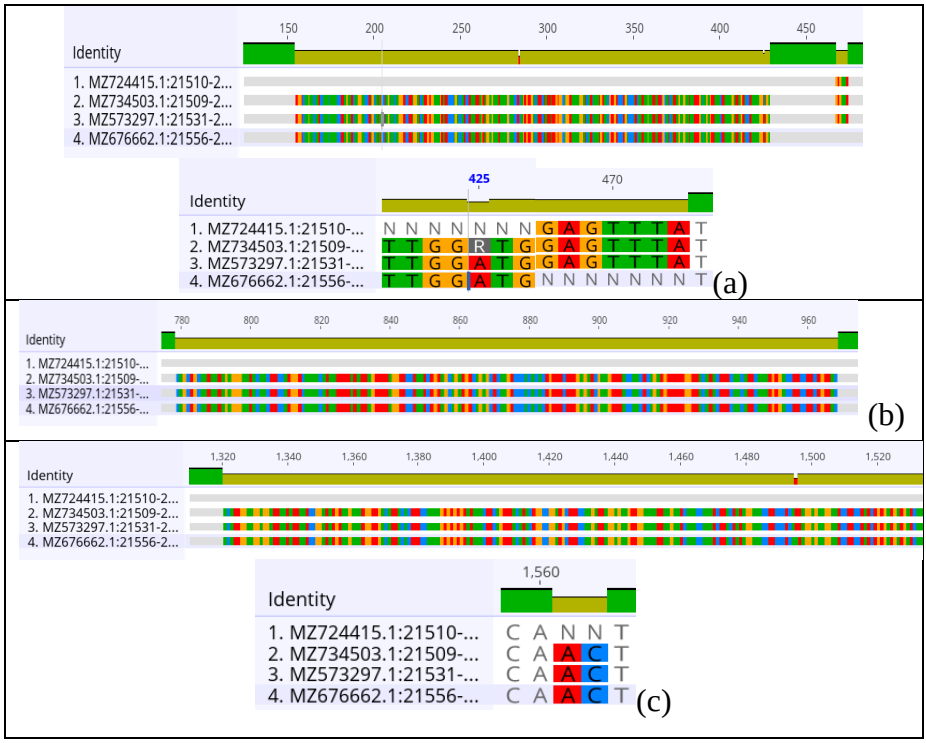


Figure 4. (a) Nucleotide base N was found in the location of nucleotide bases 155-428, and 467-473 in India sequence, and nucleotide base R in 425th nucleotide base location of the sequence from USA. (b) Nucleotide base N were found in India in the location of nucleotide bases 779-968. (c) Nucleotide base N were found in India in the location of nucleotide bases 1561-1562. Sequence alignment visualization was depicted based on the existing method (Kearse et al, 2012).

Table 3 shows the mutation pattern of each sample. India sequence has much an ambigous nucleotide base (N) so that it is make the kinship of the sequence is the most different and farest from other sequences, such as Pakistan, Uzbekistan, and USA.

Mutations occur at the 284th (C to T) nucleotide base location in Pakistan sequence which described in Figures 3a, nucleotide base position 1020th (T to C) described in Figures 3b, 1495th (A to T) described in Figures 3c, and 2036th (G to A) which described in

figure 3d, and 2542nd (A to G) which described in figure 3e. There is also an ambiguous nucleotide base (N) at the 467-473 nucleotide base sequence (figure 4a).

For the Indian sequence, there are N nucleotide bases in the 155-428 sequence (figure 4a), and there are N nucleotide bases in the 467-473 nucleotide base sequence (figure 4a) along with Bangladesh, 779-968 (figure 4b) and 1321 -1547 (figure 4c), then continued with 1561-1562 (figure 4c). N nucleotide base has a meaning that between A, C, G, T base nucleotides and also can be defined as an ambigous nucleotide base (Sidauruk, Harwati, Prasetyo, & Wirastini, 2018).

The rarest mutation occurred in the Uzbekistan sequence compared to the others, namely from the nucleotide base G (guanine) to T (thymine) that locate in the 3183rd nucleotide base (figure 3f). At 425th nucleotide base location (figure 4a), Uzbekistan and Pakistan have the same nucleotide base, which were G (guanine), while in the USA sequence there is a R nucleotide base that equal to guanine (G) or adenine (A) which are both purine bases (Sidauruk, Harwati, Prasetyo, & Wirastini, 2018).

4. Primer design results

Table 4. Primer design result for SARS-CoV-2 from India (accession number: MZ724415.1). Primer design was depicted from the existing method (Tambunan et al., 2006).

Criteria	Forward	Reverse
Sequence	ATGTCCTTCCCTCAGTCAGC	ACCAGTGTGTGCCATTTGAA
Length	20	20
%GC	55	45
GC clamp	2	1

Tm (°C)	56.16	56.64
Self-complementarity	0	0
Self 3' complementarity	0	0
Self-dimer (kcal/mol)	0	0
Hairpin (kcal/mol)	0	0
Runs (# of pairs)	3	3
Repeats (# of bases)	0	3
Cross dimer (kcal/mol)	-4.53	
Product length	160 bp	

Table 5. Primer design result for SARS-CoV-2 from Pakistan (accession number: MZ676662.1). Primer design was depicted from the existing method (Tambunan et al., 2006).

Criteria	Forward	Reverse
Sequence	ATGTCCTTCCCTCAGTCAGC	ACCAGTGTGTGCCATTTGAA
Length	20	20
%GC	55	45
GC clamp	2	1
Tm (°C)	56.16	56.64
Self-complementarity	0	0
Self 3' complementarity	0	0
Self-dimer (kcal/mol)	0	0
Hairpin (kcal/mol)	0	0
Runs (# of pairs)	3	3
Repeats (# of bases)	0	3
Cross dimer (kcal/mol)	-4.53	
Product length	160 bp	

Table 6. Primer design result for SARS-CoV-2 from Uzbekistan (accession number: MZ573297.1). Primer design was depicted from the existing method (Tambunan et al., 2006).

Criteria	Forward	Reverse
Sequence	ATGTCCTTCCCTCAGTCAGC	ACCAGTGTGTGCCATTTGAA
Length	20	20
%GC	55	45
GC clamp	2	1
Tm (°C)	56.16	56.64
Self-complementarity	0	0
Self 3' complementarity	0	0
Self-dimer (kcal/mol)	0	0
Hairpin (kcal/mol)	0	0
Runs (# of pairs)	3	3
Repeats (# of bases)	0	3
Cross dimer (kcal/mol)	-4.53	
Product length	160 bp	

Table 7. Primer design result for SARS-CoV-2 from USA (accession number: MZ734503.1). Primer design was depicted from the existing method (Tambunan et al., 2006).

Criteria	Forward	Reverse
Sequence	ATGTCCTTCCCTCAGTCAGC	ACCAGTGTGTGCCATTTGAA
Length	20	20
%GC	55	45
GC clamp	2	1
Tm (°C)	56.16	56.64
Self-complementarity	0	0
Self 3' complementarity	0	0
Self-dimer (kcal/mol)	0	0
Hairpin (kcal/mol)	0	0
Runs (# of pairs)	3	3
Repeats (# of bases)	0	3
Cross dimer (kcal/mol)	-4.53	
Product length	160 bp	

Those sequences are the best primer among the other candidates (Table 4-7). They have the same result because they have the same primer. That is caused because the sequences are similar and come from the same pangolin, which is the B.1.6.1.7.2 (delta variant). Based on the primer, India has a slightly different primer from the others because India is the farthest kinship based on the alignment and phylogeny tree construction. Hence, India becomes the outgroup in the phylogeny tree.

Primer design of PCR had done by *in silico* method using Primer3 and NetPrimer and for the results had been shown in table 4 to 7. All of the sequences (from India, Pakistan, Uzbekistan, and USA) produce 160 bp of the amplicon. The forward sequence is ATGTCCTTCCCTCAGTCAGC, while the reverse sequence is ACCAGTGTGTGCCATTTGAA.

According to Utomo, Ichsan, & Putri (2019), the criteria of the ideal primer are GC content should be around 40-60%, GC clamp should be lower than 5 bases, have 18-30 bases, and has the difference of Tm lower than 5. Generally, the Tm of the primer between 42-65°C , which the ideal primer has to be in 52-58°C, when the Tm of the primer is more than 65°C, it will reduce the annealing efectivity of the primer so that the DNA amplification process doesn't work well (Yustinadewi, Yustiantara, & Narayani, 2018). The existence of GC at the 3' end of the primer greatly helps the stability of the bond between the primer and the DNA template which is required for the initiation of DNA polymerase (PCR process). The designed primer has 20 bp

length, 55% GC for the forward and 45% GC for the reverse also have 2 GC bases in the GC clamp in the forward sequence, 1 GC base in reverse sequence that can be defined as the ideal primer. If the GC clamp has more than 5 GC bases, the primer specificity will decrease because A or T nucleotides are more tolerant of mismatches than G or C (Popp and Bauer, 2015). The Tm can be computed manually with the formula Tm = 2(A+T) + 4(G+C) also can be used to set the annealing temperature in the PCR (Borah, 2011). Melting temperature (Tm) has a range of 2 to 4°C and above 60°C to produce good PCR product (Utomo, Ichsan, & Putri, 2019). The designed primers has a good Tm, which are 56.16°C for the forward primer, and 56.64°C for the reverse sequence, the difference of them is 0.48°C which means still lower than 5°C.

Basically, the ideal primer has a length between 18 to 30 base pairs, which is enough to bond the template in the annealing temperature and gain the specific sequence. If the length of the primer too short, it can decrease the specibility of the primer so that the template will be sticked to the undesired annealing temperature. Whereas if the length of the primer too long, it cannot affect the specifity well (Borah, 2011). The length of the primers that designed is still in the acceptable range.

The absence of self complementarity and self 3' complementarity are one of the criteria of ideal primer. Self complementarity can cause a stable hairpin structure with only 4 GC base pairs at the ends and the middle of the primer (Sasmitha, Yustianara, & Yowani, 2018). As a

results, self complementarity and self 3' complementarity cannot be found.

Repeats are nucleotide that repeat in primer, which their presence causes undesired primer attachment (mispriming). The running of 4 or more bases, or repetition of dinucleotides in the ideal primer should be avoided (Yustinadewi, Yustiantara, & Narayani, 2018). In the design results, it was found that there were 3 bases of runs both forward and reverse and 3 bases of repeats on reverse, while no repeats were found on forward.

The others criteria of the ideal primer are secondary structures (self dimer, cross dimer, and hairpin) should be avoided because the existence of secondary structures can interfere the primer attachment process to the template in the PCR process (Borah, 2011). Dimer structure in the primer significantly affects the PCR primer performance, dimer is divided to self dimer and cross dimer. Self dimer is primer that binds to other primers of the same type, whereas cross dimer is primer that binds to their pairs (reverse and forward). Hairpin is the intramolecular interaction of the primers which is prohibited (cannot be accepted) in the ideal primer. For the hairpin, it is too difficult to avoid the hairpin, however the hairpin that has ΔG less than -5 kcal/mol in the 3' and less than -6 kcal/mol in the internal still be toleranted. Hairpin in the designed primer was not found in both forward and reverse primers (Sasmito, Kurniawan, & Muhimmah, 2014). For the dimer structure, there are no self dimer in the designed primer, yet there is -4.53 kcal/mol of cross dimer, which

means that the primer still can be tolerated. The ideal primer should not have a dimer structure, and primer cannot bound with their pairs (forward and reverse) which called cross dimer. The ΔG of the cross dimer lower than -5 kcal/mol still can be toleranted (Yustinadewi, Yustiantara, & Narayani, 2018).

Specifity analysis was done using BLASTN and the result is all of the samples can detect all the variants of SARS-CoV-2 virus with the whole genome. In the other side, PCR primers cannot clearly detect certain variants and mutations such as delta variants (B.1.6.1.7.2) because primers are generally used to detect the presence of viruses (Miftakhurohmah, Suastika, & Damayanti, 2013). Mutation studies are needed for a new primer design that more accurate because the primers currently designed use the original Wuhan variant, while the delta variant appeared in October 2020 that first identified in India. PCR could not detect the types of the variant therefore Whole Genome Sequencing (WGS) analysis is needed to detect for the new mutations pattern (Comas et al., 2011).

CONCLUSION & FUTURE OF STUDY

The successful of PCR process depends on the quality of primer. The best quality of the primers are achieved if the criteria of the best primers of PCR (like 18-30 bp length, 40-60% GC, difference of Tm <5, low self-complementarity, low 3' self-complementarity, and no presence of hairpins) are fulfilled, and the primer works well if can recognize the organisms and their genes according to the expected target (has high specificity and cannot stick with other organisms).

The primer that designed using in silico method yields 160 bp for the primer from India, Pakistan, Uzbekistan, and the USA are being fulfilled the criteria. All the samples from 4 different countries are the same because they have the same pangolin (B.1.6.1.7.2), and the samples are significantly similar although have slight mutations in a particular sequence after being done multiple sequence alignment (MSA) and phylogenetic tree construction analysis. Based on the specifity of the primer, all of the designs cannot clearly detect delta variant (B.1.6.1.7.2) because primer focuses on the detecting the presence of virus. For the future study, the dry lab experiment (*in silico* method) needs to be validated in the wet lab to gain the best results.

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LIST OF ABBREVIATIONS

bp	Base pair
cDNA	Complementary Deoxyribonucleic Acid
COVID-19	Coronavirus Disease 2019
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Triphosphate
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
Tm	Melt temperature
A	Adenine
T	Thymine
G	Guanine
C	Cytosine

CHAPTER 3

COMPUTER-ASSISTED COGNITIVE REHABILITATION IN ROLANDIC EPILEPSY

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INTRODUCTION

Rolandic epilepsy (RE) is the most common idiopathic focal benign epilepsy syndrome of childhood, characterized by seizures that begin in childhood and go into remission in adolescence. It has been reported to account for 8% to 25% of all cases of childhood epilepsy between the ages of 5 and 14 years (Currie et al., 2018).

The age of onset of seizures ranges from 3 to 13 years. Seizures present as twitching, numbness or tingling in the child's face or tongue, preventing speech and causing drooling. Seizures spread and become generalized seizures. In most cases, RE attacks are infrequent and usually only occur at night. These seizures typically last no more than two minutes and the child does not lose consciousness (Gascoigne et al., 2017).

Although RE is defined as a benign type of partial epilepsy of childhood that does not cause neurological defects, it has been found to impair neurocognitive functions in various studies (Fejerman, 2008).

Although electroencephalography (EEG) findings in RE are seen in the centrotemporal (rolandic) region, some studies have found impairments in planning, repetition, motor and speed regulation similar to frontal lobe-derived disorders in children diagnosed with RE (Bourel-Ponchel, Mahmoudzadeh, Adebimpe, & Wallois, 2019; Gascoigne et al., 2017; Varesio et al., 2020). In addition, it has been reported that selective and divided attention dysfunctions are observed

in children diagnosed with RE. Cognitive impairment was found to be more common, especially in those whose seizures started before the age of 8 years and who had frequent and multifocal anomalies on the EEG (Duma et al., 2021; Lindgren et al., 2004; Ramos, Coelho, Ribeiro, & Lopes, 2021; Varesio et al., 2020; Wickens, Bowden, & D'Souza, 2017).

The general opinion is that antiepileptic drug treatment is not necessary, provided that the family is well informed, because the frequency of seizures is low in RE, the seizures occur during sleep, and the seizures tend to end spontaneously before the age of 16 regardless of whether they are treated or not.

Many children do not take any anti-seizure medication for RE, and seizures usually stop by early adolescence. He or she may prescribe levetiracetam or oxcarbazepine if the child has seizures during the daytime, if the seizures disrupt sleep at night, or if the child has a reading disorder that may be associated with RE.

However, due to the fact that cognitive impairment has been shown in children with RE in some studies conducted in recent years, it has been argued that RE may not actually be a 'benign' syndrome as it is thought, and the subject of treatment has been brought up for discussion again. However, another view is that the antiepileptic drugs given may also affect the cognitive functions of the child. For example, although the systemic side effects of frequently used carbamazepine, oxcarbazepine and levetiracetam are relatively low, it has been reported that they have negative effects on cognitive

functions (Ahadi, Nasiri, Ghazavi, Mosavian, & Mansouri, 2020; Gerstl et al., 2021; Kessi et al., 2021).

1. ASSESSMENT OF COGNITIVE FUNCTIONS

Children with epilepsy may have impairments in one or more of various mental domains such as memory, learning, attention and/or executive functions (Kolar, Pejcochova, Horak, & Oslejskova, 2020; Troitskaya, Badalyan, Surkova, & Krakhalev, 2020). The methods that are frequently used to evaluate cognitive functions in children are as follows (Ayaz, Karakaya, Ayaz, Kara, & Kutlu, 2013; Kwon, Seo, & Hwang, 2012).

Bender Gestalt Visual Motor Test: This test, which is used to detect cognitive pathology on the basis of visual motor coordination skills in adults, is a reliable test that is frequently used in cognitive-developmental evaluation in children (Shakeri, Bidaki, Mirhosseini, & Kiani, 2021; Shaughnessy, 2018). Test duration is approximately 20 minutes (Tafti, Azizi, & Mohamadzadeh, 2021).

Benton Visual Retension Test: Visual attention on the basis of visual memory serves to detect cognitive pathology in short-term visual memory functions. Test time is approximately 15 minutes (Arrthy, Saravanan, & Atha, 2020; Segabinazi et al., 2020). For detailed evaluation, it is recommended to be applied together with the Bender Gestalt Visual Motor Test (Lam, Williams, Ashla, & Lee, 2021).

Mini-Mental State Pediatric Examination (MMSPE): This test, which is frequently used to detect dementia and cognitive-organic

pathologies in adults, is an easy-to-apply and short-term reliable neuropsychological scale to evaluate cognitive skills in pediatrics (Cainelli et al., 2020; Scarpa, Toraldo, Peviani, & Bottini, 2017).

Addenbrooke Cognitive Examination Test (ACE-R): It is a more comprehensive neuropsychological assessment test compared to Mini-Mental State Examination (MMSE), which is used to detect cognitive pathologies. It consists of 5 subsections and 26 items covering attention/orientation, memory, verbal fluency, language and visual-spatial functions. It also includes the MMSE. When the test is completed, it is a useful test in that it gives a total score with subscores of different cognitive domains, including the MMSE score. The maximum total score is 100. (Pan, Wang, Huang, Huang, & Guo, 2021; Starowicz-Filip et al., 2021).

Digit Span Test: This test is one of the most commonly used attention/short-term memory tests. The test consists of two parts. At the beginning, the person is asked to repeat the numbers told to him in the same order and then repeat from the end to the beginning. For both parts of the test, the span before two consecutive incorrect answers is recorded (Khan et al., 2021).

Stroop Test: It is are among the neuropsychological tests that reflect the activities of the anterior region of the brain. They are tests that measure the brain's ability to direct attention, conceptual flexibility, and the processing speed of the mind. (Koganti et al., 2021).

2. COGNITIVE REHABILITATION

Cognitive rehabilitation addresses many cognition areas such as memory, perception, comprehension attention, reasoning, concentration, communication, motor planning, problem solving and awareness (Gontkovsky, McDonald, Clark, & Ruwe, 2002). The aim is to increase functionality in all areas of family and social life and to increase the degree of independence in daily life by increasing the capacity of processing and interpreting information. In this way, people's quality of life is also increased (Kaldoja et al., 2015; Robatmili, 2019).

Rehabilitation principles in this field are mainly consolidated knowledge from neuropsychology, cognitive psychology and educational psychology, and traditional rehabilitation disciplines. Acquiring cognitive skills is very difficult and cognitive rehabilitation is a very slow process (Gontkovsky et al., 2002; Long, 1987).

Many clinicians have recommended computers as an effective tool in cognitive rehabilitation for more than a decade because of their flexibility and cost-effectiveness (Park et al., 2019; Robatmili, 2019; Oliver et al., 2017). The stimuli presented by computers can be attractive, bright and colorful and help to attract and focus the patient's attention. The computer provides immediate feedback in a clear, consistent and non-judgmental way. In addition, some patients cite working with computers as a new and enjoyable experience that improves their motivation and thus educational outcomes Park et al.,

2019; Robatmili, 2019; Oliver et al., 2017; Simone, Viterbo, Margari, & Iaffaldano, 2018).

In the following section, we will examine the effects of computer-assisted game-based cognitive rehabilitation in a child with Rolandic epilepsy.

Case Example

In this part, an 11-year-old girl who was diagnosed with Rolandic epilepsy five years ago was presented. She has been receiving medical treatment (oxcarbazepine) (Trileptal 60 mg/ml oral suspension) for 5 years. She is currently regularly using 10 ml of Trileptal 60 mg/ml per day.

Computer-assisted cognitive rehabilitation was applied to the patient in order to improve skills such as attention, memory, visual skills, and logical operations.

She was evaluated with ACE-R for cognitive status, Stroop test for selective attention and Quality of Life in Epilepsy Scale (QOLIE-31) for health-related quality of life at the beginning and after cognitive rehabilitation program.

A demo free version of game-based, computer-assisted MentalUP software was used for cognitive rehabilitation. MentalUP is an application that helps children develop mental skills such as attention, memory and concentration. It offers a daily exercise plan tailored to each individual.

The patient used this application every day for 8 weeks, 20 minutes a day. The daily rehabilitation program to develop attention, memory, logic, visual and verbal skills consists of 10 cognitive exercises of increasing difficulty (Figure 1). In addition, it was noted that the patient did not have any seizures during this period.

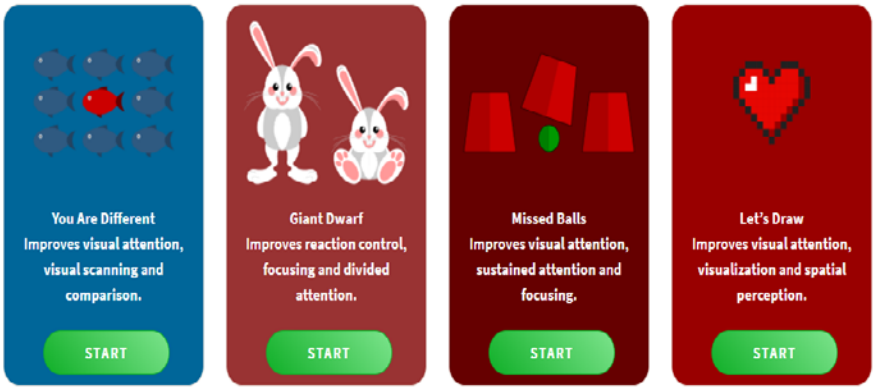
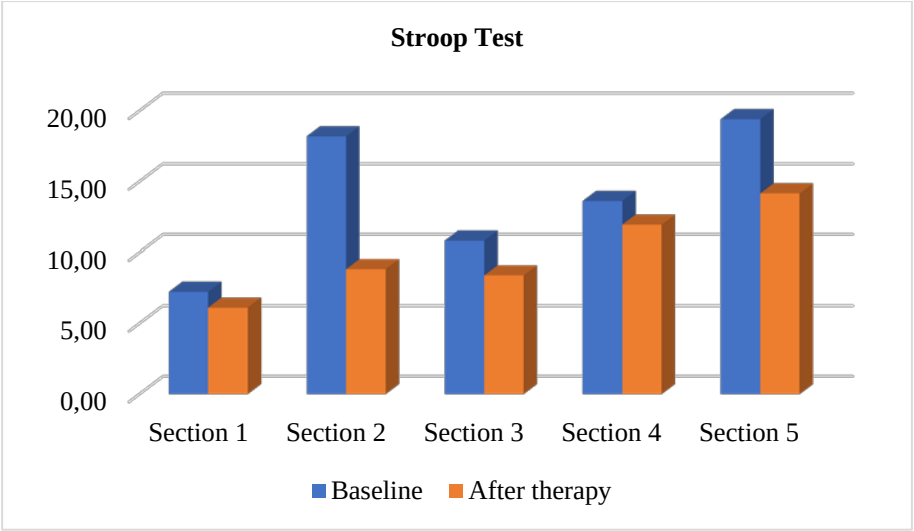


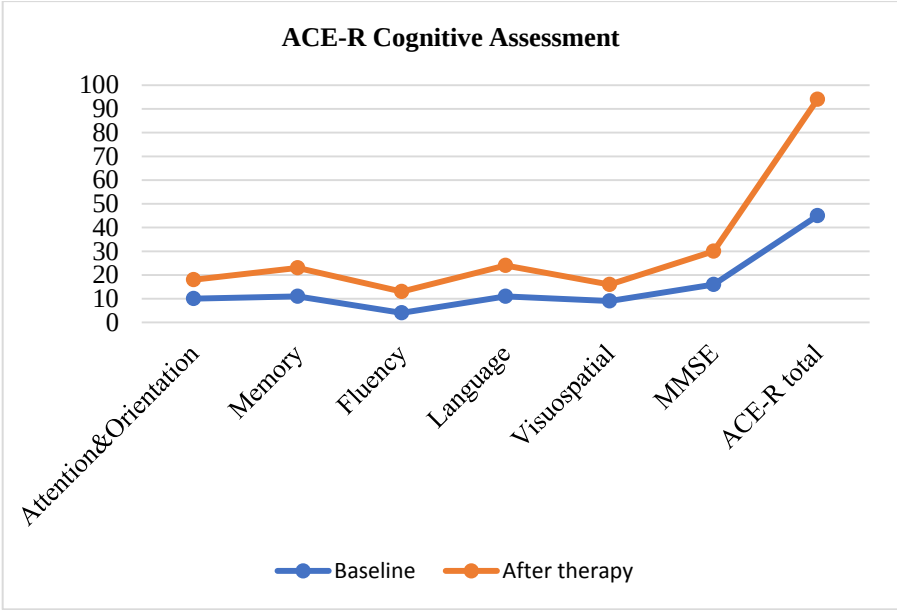
Figure 1. Some exercise examples from the MentalUP (Receive from: https://www.mentalup.co/blog/adhd-treatment-with-or-without-medications; August 24, 2021)

Before cognitive rehabilitation program, she completed the 1st part of the Stroop test in 7.24 seconds., the 2nd part in 18.21sec., the 3rd part in 10.83 sec., the 4th part in 13.63 sec. and the 5th part in 19.42 sec. After rehabilitation, she completed all parts of Stroop test in a shorter time. The first part of the test was completed in 6.13 sec., the second part in 8.85 sec., the third part in 8.40 sec., the 4th part in 11.95 sec. and the 5th part in 14.16sec.



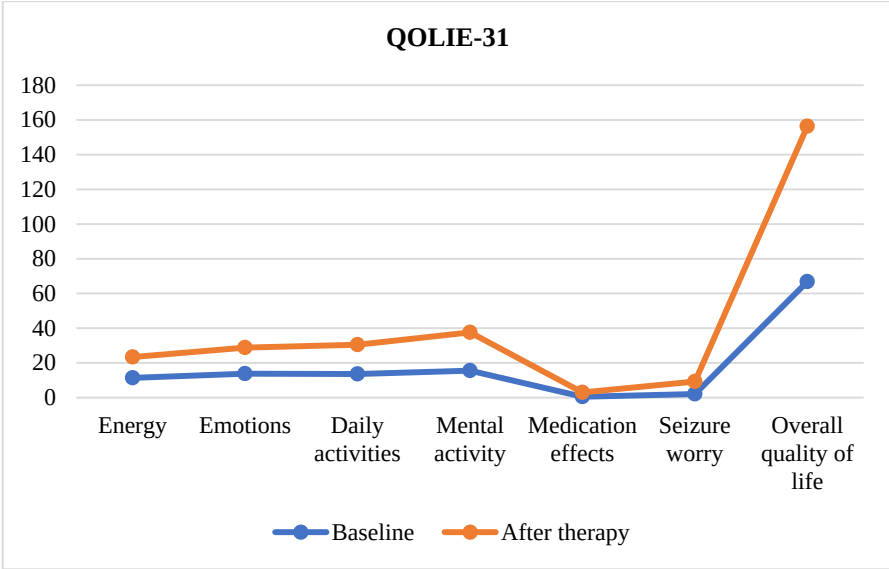
Graphic 1. The re sults of attention test

After cognitive rehabilitation, an increase occurred in all sub-parameters of the ACE-R test, including attention and orientation, memory, fluency, language, visuospatial section and MMSE sections, and in the total ACE-R score. While ACE-R total score was 45 points at the beginning, it increased to 94 points after rehabilitation (Graphic 2).



Graphic 2. The results of cognitive assessment

All sub-parameters of the QOLIE-31, including energy, emotions, daily activities, mental activity, medication effects and seizure worry, and total QOLIE-31 scores increased after cognitive rehabilitation. QOLIE-31 total score was 66.80 before rehabilitation, it increased to 89.54 points after therapy (Graphic 3). It was observed that cognitive functions and health-related quality of life improved in this patient thanks to cognitive rehabilitation.



Graphic 3. The results of epilepsy-related quality of life

CONCLUSIONS

Cognitive rehabilitation improves mental skills such as attention, memory, problem solving, visual intelligence and verbal intelligence. It also provides an increase in health-related quality of life.

There is only one study in the literature investigating the effectiveness of cognitive rehabilitation in children with partial epilepsy. In this study, a computer-assisted rehabilitation program focusing on visual organization, visual attention, and visuospatial perception was applied to 17 children with partial epilepsy between the ages of 8-12, 2 sessions a week for 5 weeks. The results of the study showed an increase in the cognitive skills of the children after rehabilitation.

We think that cognitive training should be included in the treatment process of patients diagnosed with epilepsy. Controlled studies with long follow-up are needed to generalize the results.

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CHAPTER 4

CEREBRAL PALSY

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INTRODUCTION

Cerebral palsy (CP) is a developmental disorder that occurs due to non-progressive damage to the brain before, during or after birth. Although the development period of the brain is accepted as the first 18 months, cases up to 6 years of age and which are not clinically progressive are also defined as CP (Yalçın S. et al, 2000). In general, there are problems with delayed onset of movement, gait disturbance, increased spasticity, co-contraction, insufficient power generation, and difficulty in maintaining posture. In addition, sensory, cognitive, perception, behavior, vision, speech, swallowing, epilepsy and secondary musculoskeletal problems may accompany wih CP (Rosenbaum P. et al, 2006).

1. EPIDEMIOLOGY

Although there are differences between countries in the world, the frequency of CP has been shown to be 1.5-2.5/1000 in general (Drougia A. et al, 2007). In an epidemiological study conducted in Turkey, the frequency was found to be 4.4% among children between the ages of 2 and 16 (Serdaroğlu A. et al, 2006).

In recent years, especially in developed countries, an increase in the rate of diagnosis of CP has been observed in parallel with the increase in the chance of survival of premature babies due to the earlier diagnosis and improved treatment opportunities with advancing technology. The reason for the high prevalence in studies conducted in Turkey is attributed to reasons such as the excess of consanguineous marriages, especially in rural areas, the difficulty of pregnancy

process and birth conditions, infections encountered in this period, disruptions in postpartum care, and the excess of febrile and infectious diseases in infancy and early childhood (Rodda JM. et al, 2004).

2. ETIOLOGY

Etiology in CP is considered multifactorial and usually no etiologic cause can be found. While it can be seen in prenatal, neonatal and postnatal periods of life, prenatal factors play a role in 70-80% (Yalçın S. et al, 2000). Risk factors (Matthews DJ. Et al, 1999, Shubra M. et al,2007, Berker N et al, 2005, Hamamcı N et al, 1995) ;

Prenatal Factors

- Hereditary diseases
- Multiple pregnancy
- Pregnancy infections (TORCH etc.)
- Maternal metabolic diseases (Diabetes, hypo/hyper thyroidism, pregnancy toxemia)
- Fetal posture disorders due to lack of amniotic fluid prenatal cerebral hemorrhage
- Source of intrauterine anoxia or decreased fetal blood flow (placental insufficiency, maternal hyper/hypotension, respiratory incompatibility, anemia)
- Drug use
- Rh incompatibility
- Radiation exposure in the first trimester
- Mother's habits (Alcohol, smoking, etc.)

- Developmental defects due to impaired development of brain,
- Vascular and skeletal structures
- Abortion attempts (Mechanical or with toxic, teratogenic agents)
- Abdominal trauma

Perinatal Factors

- Prematurity <36 weeks
- Low birth weight (<2500gr)
- Difficult birth
- Placental anomalies
- Birth asphyxia
- Low APGAR score
- Abnormal arrival
- Trauma
- Infection
- Bradycardia and hypoxia

Postnatal Factors

- Neonatal hyperbilirubinemia
- Neonatal infections, sepsis,
- Head trauma
- Convulsions
- Central nervous system infections
- Early childhood febrile illnesses

- Coagulopathies
- Poisonings

3. CLASSIFICATION

Classification in CP can be made according to the severity of neuropathological, etiological, clinical and functional limitation. The topographic and neurological classification adopted by the European Cerebral Palsy Surveillance Group in recent years is frequently used (Cans C et al, 2000):

1. Spastic Type (75%): Quadriplegic, Hemiplegic, Diplegic, Monoplegic, Triplegic
2. Dyskinetic Type (10–15%): Athetosis, Korea, Dystonia
3. Ataxic/Atonic Type (rare)
4. Hypotonic Type (<5%)
5. Mixed Type (10–15%)

4. CLINICAL FEATURES

4.1. Spastic Type Cerebral Palsy

Spasticity is the increase in resistance to passive movement in the extremity muscles after the upper motor neuron injury. Some upper motor involvements such as hyperreflexia, clonus, babinski and other primitive reflexes may accompany the increased muscle tone in spastic type CP. Contracture and various skeletal deformities may occur over time due to spasticity. Spasticity is prominent shoulder flexor, adductor, internal rotator, elbow flexor, forearm pronator, wrist

and finger flexor in the upper extremity and hip flexor, adductor, internal rotator, knee flexor, ankle plantar flexor, evertor, and sometimes invertor muscles in the lower extremity (Diamond M et al, 2007).

Spastic type CP accounts for 70-85% of all CP cases. Considering the affected body parts, 90% of these patients are hemiplegic, diplegic and quadriplegic cases, while 10% are triplegic and monoplegic cases (Diamond M et al, 2007).

4.1.1. Spastic Hemiplegic Type Cerebral Palsy

It occurs as a result of the lower and upper extremities of one side of the body being affected, and the involvement can be seen in varying degrees on the other side. Although it is difficult to understand in the first years of life, it can be diagnosed by realizing that over time, children use one extremity less and the catching reflexes continue unilaterally. The hemiparetic posture in children settles down by the age of two. Boys are affected more than girls, the right side is more affected than the left side, and the upper extremity is more affected than the lower extremity (Şimşek İ, 2007). Hemiplegic type CP is the group with the best walking prognosis among the spastic type CP group. There is mostly toe walking and sickle walking. There is difficulty with fine motor skills and coordination of the hand. Mental retardation was reported in 18% of the cases, epilepsy in 23%, and speech disorder in 20% (Şimşek İ, 2007).

4.1.2. Spastic Diplegic Type Cerebral Palsy

It is the type in which both lower extremities are more involved and the upper extremities are affected to a variable degree. Typical diparetic gait is present with flexion, adduction and internal rotation of the hips, flexion or full extension of the knees, and plantar flexion and inversion of the ankle. Strabismus or vision defisits are seen in 45% of spastic diplegic patients with frequent motor impairments, epileptic seizures are seen in 25%, and mental retardation is rare (Yakut A, 2006).

4.1.3. Spastic Quadriplegic Type Cerebral Palsy

Spastic type is the most severe form of CP with involvement of all four extremities. This type of involvement is usually asymmetrical with birth asphyxia or prematurity. Head-holding balance and sitting balance are impaired, and functional ambulation is often not achieved. In addition to musculoskeletal complications such as hip dislocation, scoliosis, severe spasticity and contracture, mental retardation, strabismus, oromotor problems and seizure history are also present. Difficulty in swallowing and speaking due to involvement of the corticobulbar tract, difficulty in feeding due to involvement of the oropharyngeal muscles, malnutrition and aspiration pneumonia may occur (Yakut A, 2008).

4.2. Dyskinetic Type Cerebral Palsy

It is a type of CP characterized by abnormalities in balance and coordination of voluntary movements, posture control and muscle tone. It is also called extrapyramidal CP. It is due to basal ganglia damage that develops after severe asphyxia or hyperbilirubinemia.

While hypotonia is seen in the first years of life, movement disorder occurs after about two years of age. Long duration of hypotonia predicts that the involvement may be severe. While sleeping, muscle tone is normal and there are no involuntary movements. Abnormal movement patterns such as athetosis, chorea, ballismus and dystonia develop in case of any active movement attempt. These movements may increase in fatigue, stress, insomnia, and may change during the day. Speech disorder occurs due to the involvement of the facial, tongue and vocal cord muscles. Although there is usually no mental retardation, it can be thought that there is a mental disorder due to problems such as hearing loss and speech disorder (Swaimann KF et al, 1999).

Athetosis: Slow, curvy, involuntary movements in which both agonist and antagonist muscles are actively contracted, especially in the distal extremities.

Chorea: Sudden, irregular, jumping movements usually seen in the head, neck and extremities.

Choreetetoid: It is a combination of athetosis and choreiform movements. They are generally large-amplitude, involuntary movements.

Dystonia: Slow, rhythmic movements with varying tone (Molnar GE, Alexander MA, 1999).

4.3. Ataxic Type Cerebral Palsy

It is the type of CP in which balance coordination disorder is observed especially when walking due to cerebellum damage. In this type, in which hypotonicity is dominant in the first years of life, ataxia occurs while the tone improves after 2-3 years of age (Shubra M. et al,2007). There is weakness in typical ataxic gait and fine motor skills. Dynamic tremor, explosive speech, Romberg's sign, nystagmus, mental retardation and asterognosis can be counted among the accompanying problems (Dursun N, 2004).

4.4. Hypotonic Type Cerebral Palsy

It is often a transitional stage in the development of athetosis or spasticity. Although ataxic type CP is seen in most of the cases, generalized hypotonia may become permanent in some cases. It is manifested by decreased muscle tone at rest, decreased stretch reflexes, and a decrease in primitive reflex patterns (Molnar GE, Alexander MA, 1999).

4.5. Mixed Type Cerebral Palsy

In this type, which constitutes 10% of patients with CP, both spastic and dyskinetic types are seen together. The coexistence of athetosis and spastic diplegia is more common. Increased muscle tone and involuntary movements may coexist due to damage to the pyramidal

and extrapyramidal regions. The association of choreatetosis and spasticity is observed more rarely (Bangash AS et al,2014).

5. PROBLEMS ACCOMPANYING CEREBRAL PALSY

Mental Retardation: Its incidence is 23-44%. It is observed more frequently in those with low birth weight and a history of prematurity and in quadriplegic cases, while it is less common in ataxic and hemiplegic cases (Odding E et al, 2006).

Epilepsy: Approximately half of the patients with CP have a history of epileptic seizures. Central nervous system malformations, infections and gray matter damage in prenatal, perinatal and postnatal periods increase the risk of epilepsy. Seizure onset age is usually in the first two years. While quadriplegic and hemiplegic types are observed more frequently and resistant in CP, their incidence is less in diplegic and ataxic type. Although different epileptic forms are seen according to the type and severity of the disease, generalized or partial seizures are often observed. Grand-mal epilepsy in which tonic-clonic contractions are seen in quadriplegic type CP and focal motor contractions are more common in hemiplegic type CP (Wallace SJ, 2001).

Swallowing Disorders: In patients with CP, 57% of sucking and 38% of swallowing problems are seen in the first year (Odding E et al, 2006). Difficulty in sucking, chewing and salivation, disorder in lip and tongue movements, insufficiency in pharyngeal muscles and increased gag reflex cause swallowing and feeding disorders in

patients. While feeding problems create growth and development problems in early childhood, poor grinding of nutrients can cause aspiration.

Drooling is seen in about 10% of children with CP. It may occur in cases such as head-holding balance disorder, weakness or loss of sensation in the facial muscles, and mouth breathing. In addition to increasing susceptibility to infection such as aspiration pneumonia, it is one of the factors affecting the child's care and integration into social life (Erkin G et al, 2005).

Speech Disorders: In CP, there may be dysarthria due to involvement of the corticobulbar pathways, articulation problems due to disorders in the oromotor muscles, and phonation problems due to involvement of the larynx muscles. Speech disorders are seen in 95% of dyskinetic type, 85% of spastic quadriplegic type, 30% of spastic hemiplegic type and 20% of spastic diplegic type (Odding E et al, 2006).

Gastrointestinal Problems: The most common gastrointestinal problems in CP are gastroesophageal reflux and constipation. Chronic constipation is observed in patients with whole-body involvement and immobilization, and those who cannot take adequate fibrous and liquid foods due to swallowing disorders or oromotor problems (Dormans JP et al, 1998).

Vision Problems: Approximately half of children with CP have vision problems. Decreased visual acuity is seen in 71%, strabismus in 25% and hemianopsia in 15%. Strabismus is observed more frequently

in diplegic and quadriplegics, and hemianopsia and visual perception problems are observed more frequently in hemiplegics (Odding E et al, 2006).

Hearing Problems: Hearing loss is observed in 3-10% of children with CP. It is more common in patients with a history of prenatal infection (TORCH etc.), neonatal asphyxia and hyperbilirubinemia (Dormans JP et al, 1998).

Respiratory Problems: Insufficient coughing, impaired pulmonary ventilation due to weakness in respiratory muscles, and swallowing problems cause pulmonary infection to be seen more frequently in children with CP than in healthy children (Kwon YH et al, 2015).

Urinary Problems: 25% of children with CP have urinary dysfunction. By the age of 6, continence is achieved in 54% of patients with quadriplegic involvement and in 80% of patients with diplegic and hemiplegic involvement (Odding E et al, 2006). This situation, which may be in the form of uninhibited bladder or hyperactive detrusor contractions, increases the risk of vesicourethral reflux and patients have frequent urinary tract infections. Immobilization, mental status disorder and communication problems increase urinary incontinence in patients (Silva JA et al, 2010).

6. EVALUATION OF THE PATIENT WITH CEREBRAL PALSY

Objectives in the evaluation of a child with CP; to rule out progressive neurological diseases, to determine the type of involvement, to prevent secondary complications, to determine the current functional capacity of the patient and to set realistic goals accordingly (Koman LA et al, 2004).

Since CP is a clinical disease that does not represent a single etiology, pathology and prognosis, there is no specific diagnostic test. It is a diagnosis of exclusion based on a careful history and physical examination. Early detection of the disease allows early intervention and prevention of secondary complications. While the disease does not give a clear finding other than hypotonia in the muscles after birth, it is difficult to diagnose within the first 6 months. In this period, delay in laughing, decrease in spontaneous motor activity, not loss of primitive reflexes and abnormal tone findings may help in the diagnosis. When patients reach 1 year of age, the diagnosis of CP is approached by findings such as spasticity with increase in cortex maturation, abnormal involuntary movements, increase in deep tendon reflexes, presence of pathological reflexes, and early extremity preference (Palmer FB, 2004).

When evaluating the patient, the first step is to take a detailed anamnesis, starting from the prenatal stage, including family history and potential risk factors. Problems that may be experienced during

pregnancy, complications that may occur during delivery, prematurity or low birth weight should be questioned, and it should be evaluated whether there is retardation in the motor and cognitive developmental stages of the child after birth.

After a detailed history, a detailed physical examination should be performed. Physical examination should be done separately and carefully as neurological, orthopedic and functional examinations. Neurological examination can be started with the mental state of the patient. The mental activities of the patient suitable for his age, his relationship with the family and the environment are observed. The patient's vision, hearing, comprehension and speaking abilities are evaluated. Primitive reflexes (moro reflex, palmar grasp reflex, asymmetric tonic neck reflex and tonic labyrinth reflex) are evaluated. While these reflexes are gradually suppressed during cortex maturation, they should be replaced by postural reactions such as parachute and balance-correction reactions. Motor impairment should be considered if primitive reflexes are delayed or persisted, and postural reactions are delayed or absent (Blasco PA, 1994). Another condition that should be considered in the neurological examination is the presence of muscle tone and involuntary movements. Muscle tone is determined by evaluating the amount of resistance during the patient's range of motion. While hypotonia is observed in the first years of life, over time, a pronounced increase in tone is observed. A fluctuating course of tone is a finding in favor of dyskinetic CP.

In orthopedic examination, waist, back, upper extremity joints (shoulder, elbow, wrist and fingers), lower extremity joints (hip, knee, ankle and fingers) should be evaluated separately. Deformities such as spinal problems (scoliosis, lordosis, kyphosis, etc.), posture and balance-coordination disorders, joint subluxation, spasticity and contracture should be investigated (Chan G, Miller F, 2014). In the functional examination, the patient's head holding, sitting, trunk, standing, walking and hand skills should be evaluated.

There are various scales that evaluate functionality in SP. Gross Motor Function Measurement (GMFM) is a scale that validity and reliability have been demonstrated in children with CP aged 5 months to 18 years, showing motor functions. One of its important advantages that it can be used in every period starting from infancy and that it reveals the developments in childhood (Normark E et al, 1997). Another scale used in functional assessment is the Functional Independence Measure (WeeFIM). This scale, which was designed for adults and later adapted to pediatric patients, is used in children aged 6 months to 7 years. In this scale, the degree of independence of children in all functional areas, including mental and social functions, can be reliably evaluated (Wong V et al, 2004). Apart from these scales, there are also similar criteria with proven validity and reliability in CP such as PEDI (Pediatric Evaluation of Disability Inventory) and MAI (Movement Assesment of Infants).

Neuroimaging is used to determine the etiology and lesion localization in patients with CP. Cranial ultrasonography is preferred because it is practical, noninvasive and reliable in the neonatal period. With cranial ultrasonography, the ventricles, basal ganglia, corpus callosum, and periventricular white matter can be evaluated. Cranial computed tomography (CT) is recommended to identify hemorrhagic lesions in patients with a low hematocrit level or a history of bleeding disorders. Magnetic Resonance (MR) imaging is used in those with suspected CP outside the neonatal period (after the earliest 2-3 weeks of life). It is preferred in terms of providing reliable, noninvasive and more accurate information than other methods in determining lesion localization and etiology (Msall ME et al,2009).

7. REHABILITATION IN CEREBRAL PALSY

CP rehabilitation should be planned according to the patient's age, type of involvement and functional status. Rehabilitation team consists of physical medicine and rehabilitation specialist, physiotherapist, occupational therapist, psychologist, rehabilitation nurse, orthotics technician, social worker, child development specialist and family. The aim of rehabilitation should be to correct abnormal posture, movement and gait patterns, to develop existing functional skills, to teach new skills, to prevent and treat secondary complications such as spasticity, joint deformities and contractures, and to provide support to himself and his family and to reintegrate into society (Stranger M et al, 2004).

Age is an important factor in CP rehabilitation and rehabilitation should be started as early as possible. Different approaches can be used in each age group. For example, to provide optimal posture in the infant period, to provide skills such as eating and drinking; to develop ambulation, fine motor skills, cognitive and communication abilities in the preschool period; It should be aimed to increase social skills in school age and adolescence, and to combat secondary problems such as scoliosis and contracture. While the treatment is being programmed, the family, the most important member of the team, should not be forgotten, and they should be supported in the treatment process through home programs (Solopova IA et al, 2015).

There are various methods in different structures within physiotherapy programs. While some approaches based on neurophysiological foundations have survived to the present day, some approaches have not been able to maintain their popularity. In addition to neurophysiological-based approaches, strengthening, stretching, hydrotherapy, electrotherapy, hippotherapy, bimanual therapy, restrictive compulsive motion therapy and ambulation therapies are used independently or in combination (Hartley J, 2002).

CONCLUSION

Cerebral Palsy is one of the most common causes of childhood disability. Children should be carefully examined in terms of all systems as well as the musculoskeletal system, necessary precautions should be taken before complications occur. Early diagnosis and rehabilitation are positive prognostic factors of the disease.

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CHAPTER 5

INTRANASAL IN SITU GEL AS A PROMISING APPROACH FOR ENHANCING BIOAVAILABILITY AND BRAIN DELIVERY

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INTRODUCTION

In-situ gelling systems initially exist as solutions and gel depending on various factors when they come into contact with tissues (nasal, ocular, vaginal exc.). In situ gels are drug delivery systems that are solutions before administration, but during administration they undergo gelation in situ, to form a gel. They are capable of forming gels in response to different endogenous stimuli, such as temperature increase, pH change and the presence of ions (Vigani et al. 2020). These systems have many advantages such as ease of administration, prolonged residence time and sustained drug release at the application area, with a reduction in administration frequency and an increase in patient compliance and comfort (Ajazuddin et al. 2012).

The nasal route is used as an alternative route to administer systemic drugs to the organism, which are generally metabolized in the gastrointestinal tract or cannot be administered orally because they have a first-pass effect in the liver. The nasal route, with its advantages such as high permeability of the nasal epithelium, wide absorption area, and rapid blood flow; It has become an alternative route of administration for many drugs for systemic effect (Alavian and Shams 2020). Nasal drug administration is a route of drug administration used when common drug delivery methods (e.g., intravenous, intramuscular, or oral) cannot be applied. Recently, many drugs have been shown to have better bioavailability with the nasal route than the oral route (Dhakar et al. 2011). It is also possible to encounter adverse effects such as mucociliary clearance, low drug

bioavailability and sudden changes in blood-plasma concentration in nasal drug administration (Erdő et al. 2018). In order to overcome such problems, in situ gelling systems applied nasally in recent years overcome these problems by not providing a controlled release (Khatri et al. 2020).

Neuropharmaceuticals are one of the areas with the highest growth potential in the pharmaceutical industry. The reason for this is the potential of the pharmaceutical market to have a large share in the pharmaceutical industry, and the fact that one out of every three individuals has a disease affecting the central nervous system (CNS) during their lifetime (Archibald and Quisling 2013). They are also one of the drug groups whose bioavailability is increased by the nasal route (Agrawal et al. 2020).

In this part of the book, brain targeting approaches of intranasal in situ gel systems will be discussed.

WHY INTRANASAL DELIVERY?

Because of convenience and effectiveness of the method. For several reasons it is convenient from patient's and pharmaceutical industries perspective (Table 1).

Table 1. Advantages of intranasal drug administration.

Convenience	Non-invasive
	Painless
	Easy performed self-medication
	Trained personnel not required
	Rapid onset
	No need for sterilization
	No need for nasal preparations

But most importantly – it's effective (Figure 1). Concentration time rate profile of drugs, administered through nasal route, and drugs, administered intravenously, is very similar (Agrawal et al. 2020).

The effectiveness of intranasal drugs is predetermined by anatomy and physiology of the nose. Nasal passage anatomically has a very large surface area and is well supplied with blood (Kaur et al. 2016). Those qualities make drug absorption and onset of pharmacologic action faster, treatment – easier and more effective.

The permeability of nasal epithelium is high because of the thin barrier between it and the network of blood vessels (Mainardes et al.

2006). Lipophilic drugs and low molecular weight drugs easy penetrate through nasal epithelium.

Another big advantage is a possibility of direct nose-to-brain delivery of the drug. Olfactory region is the only location, where neuroepithelium is directly exposed to an external environment. Such anatomical properties provide drug delivery route that circumvents blood-brain barrier, which tends to be a big limitation for many drugs (Garg and Goyal 2014).

Possibility to avoid first pass metabolism as well as gastrointestinal tract degradation (enzymatic and chemical) requires lower dose of drug, which means less side effects and lower treatment cost (Ban et al. 2018).

Figure 1. The advantages of intranasal delivery system.

There are some limitations of intranasal drug delivery as well. Simple cold or other pathological conditions – local irritation, allergies or even GERL, may alter natural properties of nasal pathway and consequently change the bioavailability of a drug. Muco-ciliary clearance negatively affects with permeability of drug. High concentrations of drug or other constituents may be harmful and injure nasal membrane. Nasal absorption of high molecular weight or polar compounds is poor (Kaur et al. 2016).

NASAL ABSORPTION – HOW DOES IT WORK?

First step – drug absorption and transportation through the mucus. As mentioned before, low molecular weight and nonpolar particles easily transfer through this layer (Figure 2).

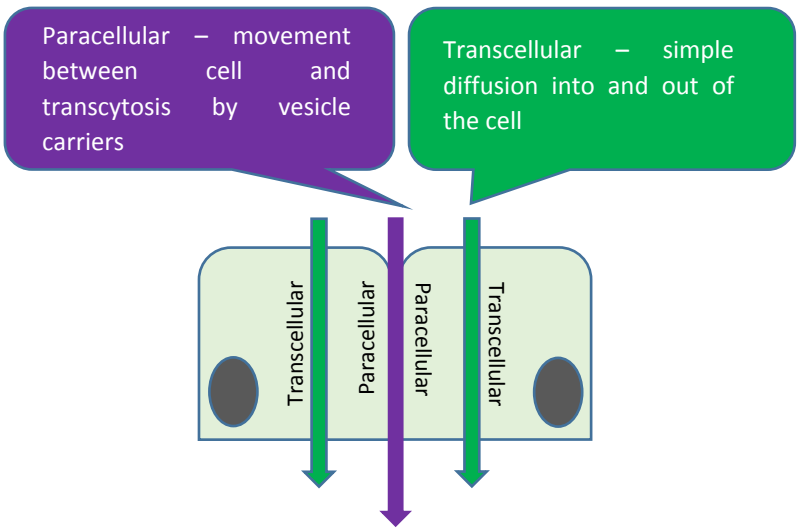

Figure 2. Mechanisms of absorption through mucosal layer include transcellular and paracellular transportation.

Kaur et al. suggested simple characterization summary of transcellular and paracellular transport mechanisms (Table 2) (Kaur et al. 2016).

Table 2. Main characteristics of transcellular and paracellular transport.

Transcellular process	Paracellular process
Lipoidal route of transport	Aqueous route of transport
Drugs can cross membranes of the cells using carrier-mediated transport through the openings of the junctions	The process between the cells and transcytosis by vesicle carrier
Lipophilic drug transport intensity is rate-dependent on their lipophilicity	Non-intense and passive route
	Sufficient for hydrophilic drugs
	Higher molecular weight of water-soluble compounds means lower intranasal absorption – with molecular weight greater than 1000 Daltons, bioavailability is poor.

There are several factors that predetermine nasal drug absorption (Table 3 and 4) (Kaur et al. 2016).

Factors related to drug

Table 3. Factors to predetermine nasal drug absorption.

Molecular weight	Increasing molecular weight leads to decreasing intranasal absorption
Chemical form	Reformation of the drug can change its absorption (for example salt to ester)
Polymorphism	Dissolution rate, solubility of the drug formulation and absorption depends on polymorphism
Solubility and dissolution rate	Particles must be dissolved before absorption
Lipophilicity	As lipophilicity increases, permeation of the drug through nasal mucosa also increases
Partition coefficient and pKa	Absorption of non-ionized species is better comparing to ionized species

Factors related to formulation

Table 4. Factors to predetermine nasal drug absorption.

PHYSIOCHEMICAL PROPERTIES	PHYSIOLOGICAL PROPERTIES
pH and mucosal irritation Optimal pH of nasal drug must be 4,5 – 6,5 – the irritation then should be prevented.	**Effect of deposition on absorption** Drug deposition in the anterior part of nasal cavity increases nasal residence time and absorption.
Osmolarity Administrating isotonic solutions results in increased permeability.	**Nasal blood flow** Drug absorption depends on vasoconstriction and vasodilatation of the blood vessels.
Viscosity Higher viscosity results in increased contact time between drug and mucosa.	**Effect of enzymatic activity** Enzymes, that are present in mucosa, affect stability of the drug.
Buffer capacity The suitable buffer capacity maintains the pH.	**Effect of muco-ciliary clearance** The muco-ciliary clearance affects residence time of the drug in the nasal cavity.
Drug concentration, dose, dose volume These characteristics influence the performance intensity of the nasal delivery.	**Effect of pathological conditions** Invasive procedures or pathological health conditions may negatively impact drug absorption through muco-ciliary transport and/or pH alterations.

There are several strategies to overcome these factors. One of them is in situ gelling system.

IN SITU GELLING SYSTEMS

Why in situ gel?

There are many drug formulations for intranasal administration (Table 5) (Ban et al. 2018).

Table 5. Examples of intranasal drugs formulations.

Nasal drops	Nasal inserts	Nasal gels	Nasal sprays
Nasal particulate system	Nasal ointments	Nasal powders	Nasal microemulsions

To overcome the limitations of nasal route, improve the bioavailability of neurotherapeutic molecules and nasal absorption, various innovative strategies such as mucoadhesive agents, nanocarrier systems, and gel-based systems have been extensively studied. According to the above mentioned challenges, related to the nasal route, the in situ gelation systems show a promising approach that prolongs the drug release, reduces the outflow of the administered dose *via* muco-ciliary clearance, increases the drug retention time in the nasal cavity, and increases the drug absorption (Table 6) (Aderibigbe 2018).

Table 6. Advantages of in situ gelling systems (Mayuri et al. 2018).

ADVANTAGES OF IN SITU GELLING SYSTEMS
Reduction of drug leaking into the back of the throat, which reduces the bad taste problem and the drug loss problem.
Reduction of drug leakage out of the anterior part of nasal cavity.
Formulation is localised on the mucosa which causes better absorption.
Additional agents can be added to reduce local irritation possibility.
Suitable for systemic and local drug delivery.
Dose precision.

Moreover, in situ gelling systems are recently developed dosage form, which exhibits sol-gel transition during administration onto mucosa in response to the conversion of the physiological environment.

Shortly, these systems have a low viscosity, transparent polymer solution. Furthermore, when these polymer solutions act with external stimulus like pH, temperature, ionic change, magnetic field, light, electrical signal or biological environment, they are converted into a viscous gel (Sosnik and Seremeta 2017).

There are many types of in situ gels. Gellan gum, alginate and pectin are used in ion sensitive in situ gelling systems because they are anionic. These polymers pass from sol to gel by cross-linking with some monovalent (Na^+) and/or divalent (Mg^{2+} and Ca^{2+}) cations found in different physiological fluids such as saliva, tears, nasal fluids.

Cation type, cation concentration, viscosity of the polymer used as the gel are important factors in the sol-gel transition rate (Mahdi et al. 2016; de Almeida et al. 2020; Jelkmann et al. 2020).

Table 7. Mechanisms of in situ gelling systems (Kaur et al. 2015).

Physiological stimuli of gelling systems	
Thermally triggered systems • Positively thermosensitive gels • Negatively thermosensitive gels • Thermally reversible gels	The transition solution-to-gel is triggered by the change of temperature.
pH-triggered systems	The transition solution-to-gel is triggered by the change of pH.
Physical mechanisms of gel formation	
Diffusion	Solvent from formulation diffuses into surrounding tissues.
Swelling	The formulation absorbs water form surroundings and swells, therefore covering the required amount of space.
Chemical reactions-based gel formation	
Ionic crosslinking	The transition solution-to-gel is triggered by the presence of various ions.
Enzymatic crosslinking	The transition solution-to-gel is induced by naturally occurring enzymes.
Photo polymerization	The transition solution-to-gel is triggered by the application of electromagnetic radiation.

Temperature sensitive in situ gels involve the development of mucoadhesive formulations comprising of polymers which exhibit temperature-triggered sol-to-gel transitions in the range of 25–37 ^{o}C. Below or above this temperature range, early or late gelation might occur, either hindering ease of handling or inducing liquid formulation's leakage in the outer region of the application site (Lien et al. 2013; Matanović et al. 2014) . Among the polymers used for this purpose are cellulose, chitosan, xyloglucan, and gelatin and their derivatives as natural polymers; as synthetic polymers poly-N isopropylacrylamide (pNiPAAm), poly(ethylene oxide)-b-poly(propyleneoxide)-b-poly(ethylene oxide) (PEO/PPO/PEO) block copolymers, poly(ethylene oxide)-b-poly (D,L-lactic acid-co-glycolic acid)-bpoly(ethylene oxide) (PEO/PLGA/PEO) triblock copolymers, and amphiphilic triblock copolymers, composed of PEO and polye-caprolactone (PCL) (PEO/PCL/PEO) derivatives are used (Matanović et al. 2014).

The pH sensitive in situ gels, consist of polymers whose sol-to-gel transformation is induced depending on the pH change. All the pH-sensitive polymers are either recipients or donors of protons when environmental pH is changing. According to the pKa, the polymers ionize with the change in pH and ultimately produce changes in polymer conformation and solubility that result in gelation. Polyacrylamide (PAA) and chitosan are the most used polymers for this purpose (Swift et al. 2016; Irimia et al. 2018). PAA is transforming sol-to-gel when pH rise from 4.2 to 7.4. At higher pH,

polymer is forming bonds with mucin which results in formation of in situ gel. However, chitosan sol form at pH 6.2, but when pH rise greater than the 6.2 form gel (Karavasili and Fatouros 2016). In addition, there are in situ gelling systems that are less used, sensitive to enzyme, sensitive to dilution and sensitive to light (Kouchak 2014).

APPLICATION OF IN SITU GELLING SYSTEMS FOR INTRANASAL BRAIN DRUG DELIVERY

In situ gel formulations are applicable for nose-to-brain delivery of various bioactive materials to treat several brain disorders (Table 8), such as migraine, epilepsy, Parkinson, also psychological disorders etc. The most abundant and intensively studied are temperature-sensitive in situ gelling delivery systems. The biomedical applications of this delivery system are discussed in this section, when various neurological disorders are targeted.

Parkinson's disease results primarily from the death of dopaminergic neurons in the substantia nigra (Dauer and Przedborski 2003). The current medications treat the symptoms. There are several choices of different kinds of medicine. Salatin et al. 2018 developed selegiline hydrochloride, loaded in situ intranasal gel for enhanced drug transport to the brain. Selegiline hydrochloride is an MAO-B38 inhibitor, which is used as anti-Parkinson drug (Dauer and Przedborski 2003). The pharmacokinetic potency of available dosage forms (disintegrating tablet, capsule and transdermal patches) is constrained due to low bioavailability and poor oral absorption

(Hidestrand et al. 2001). Salatin et al. 2018 developed selegiline hydrochloride in situ nasal gel. It was prepared by cold method using PF127 as a gelling agent. The selegiline hydrochloride release study demonstrated an initial burst release of 33.6 % for 15 min with slow and steady release of 78.8 % drug for next 8 h. The drug release depends on the nature of polymers used, viscosity and temperature of the formulation (Salatin et al. 2018).

Sharma et al. 2014 formulated levodopa loaded chitosan nanoparticles and incorporated them into in situ nasal gel to improve the bioavailability of the drug (Sharma et al. 2014). Levodopa is a widely used drug for Parkinson's disease symptoms, but poor bioavailability and peripheral degradation by decarboxylase enzyme reduce its therapeutic potency (Nyholm 2006). To avoid the current side effects, it's reasonable to use a nano-carrier systems. The formulation mentioned, was prepared using chitosan nanoparticulate system as a drug carrier, and it was incorporated into the PF 127. *In vitro* drug release study showed initial burst release with slow and prolonged release for 7 h. The obtained results were promising, the *in vivo* studies demonstrated recovery of a substantial amount of drug from rat brain (Chen et al. 2013).

Chen et al. 2013 developed the intranasal in situ curcumin gel for brain targeting, which is used to treat AD and other neurodegenerative disorders. Gelling agents used by the authors are PF 127 (20 %) and PF-68 (2 %). The *in vitro* study showed sustained release of 80 % of drug for 6 h. The *in vivo* study demonstrated higher adhesion to the

nasal mucosa, increased nasal retention time as compared with controlled group. Furthermore, no nasal irritation or muco-ciliary damage was found in the *in vivo* morphological estimation, which assures the safety of such formulation. Moreover, the significantly higher drug concentration was found in different parts of the brain upon intranasal administration route than the one observed after administration of intravenous formulation (Nazar et al. 2012)

Table 8. Examples of in situ gelling systems for intranasal delivery of CNS disorders treating agents (Agrawal et al. 2020).

CNS disorder	Drugs/carrier system
Neuroprotective	Ovalbumin/Liposome in-gel-mucosal graft
Parkinson's disease	Selegiline hydrochloride Ropinirole Levodopa/nanoparticle FF-127 and PF-68 RPN hydrochloride/NLC
Alzheimer's disease	Rivastigmine hydrogen tartarate/PLGA nanoparticle
AD	Tacrine Curcumin/hydrogel
Neurodegenerative disorder	Resveratrol/cubosome
Depression	Agomelatine
Anxiety and depression	Venlafaxine
Schizophrenia	Quetiapine fumarate/SLN
Epylepsy	Lorazepam/microsphere

Migraine	Amlotriptan maleate/SLN Zolmitriptan/nanoethosome Eletriptan hydrobromide/ethosome
AD, PD, glioma	siRNA/Dendriplex

CONCLUSION

In the last decade, smart polymer-based in situ gelling systems raised a promising drug delivery system for nose-to-brain delivery of bioactive agents to treat various neurological disorders. The polymers used in formulation of this delivery system tend to transform from solution to gel phase upon exposure to a different kind of stimulus. Studies demonstrated the addition of mucoadhesive agents improves the drug absorption through nasal epithelium by prolonging the residence time in nasal cavity. The in situ gelling systems resolves the prime limitations of intranasal drug delivery, including rapid muco-ciliary clearance, enzymatic degradation and poor permeation of hydrophilic agents.The in situ gelling systems is suitable for delivery of a wide variety of therapeutic agents like small and large size lipophilic as well as hydrophilic molecules to the brain via intranasal route.

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CHAPTER 6

THE USE OF PROBIOTICS IN GASTROINTESTINAL DISEASES

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INTRODUCTION

The human body contains microorganisms living together in a commensal manner. These microorganisms are in constant interaction with the human body. All of these microorganisms are collectively called the microbiota. Approximately 70% of the microbiota in the human body is found in the gastrointestinal tract (Aslan & Altindiş, 2017).

Probiotics were first described by Lilly and Stillwell in 1965. Today, probiotics are defined as live microorganisms that provide health benefits to the host when administered in adequate amounts (Hill et al., 2014).

When both the number and diversity in the microbiota are impaired, dysbiosis occurs. The decrease in the diversity of the microbiota and the development of dysbiosis may increase the susceptibility to various diseases. It has been suggested that replacing the missing microorganisms in the microbiota and correcting dysbiosis may be effective in preventing and/or treating the development of some diseases (Markowiak & Śliżewska, 2017). Today, the use of probiotics seems to be the most effective method for replacing the missing commensal microorganisms in the microbiota.

1. MICROBIOTA

Microbiota include many microorganisms consisting of bacteria, fungi, viruses, protozoa, and eukaryotic cells. The microorganisms in the gut make up the gut microbiota. The number of bacteria in the

human body is about 10 times greater than the number of body cells. The structure of gut microbiota differs between individuals. The most dominant bacteria in the microbiota are the Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria species. There are many causes for the differences in the gut microbiota between individuals, with the most important one in our day, our diet. The gut microbiota interact with the host, and this interaction plays a key role in the health of the individual. The microbiota demonstrates a beneficial interaction with the intestinal epithelial barrier. A relationship also exists between the microbiota and the mucosal immune system, the enteric nervous system, and the intestinal muscle. It has been demonstrated in many studies that like the interaction between the microbiota and the gut, the gut also communicates biochemically with the brain (Carabotti et al., 2015; Dinan & Cryan, 2017). When the interaction between the microbiota and the host is disrupted, potential risks for the development of many diseases arise (Sartor & Wu, 2017). Studies have shown that inflammatory bowel diseases, irritable bowel syndrome, and metabolic diseases may occur more frequently when the diversity in the microbiota in the human gut is low (Manichanh et al., 2012; Qin et al., 2012; Rajilić-Stojanović et al., 2011). The intestinal microbiota can be restructured through the use of probiotics and/or prebiotics, therapeutic diets, or antibiotics. Studies suggest that replacing the missing microorganisms in the gut microbiota may be an effective method in the prevention and treatment of microbiota-related diseases.

2. PROBIOTICS

The most prominent features when defining probiotics are that the microorganisms must be alive and provide a health benefit to the host (Hill et al., 2014). Today, the most commonly used microorganisms in probiotics are: *Lactobacillus* (*L. acidophilus, L. casei, L. fermentum, L. gasseri, L. johnsonii, L. lactis, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus,* and *L. salivarius*), *Bifidobacterium* (*B. bifidum, B. breve, B. lactis,* and *B. longum*), *Streptococcus* (*S. thermophilus* and *S. salivarius*), the nonpathogenic *Escherichia coli* strain (Nissle 1917), in addition to *Saccharomyces boulardii*, which has probiotic properties as yeast. The microorganisms to be used in probiotics must be resistant to stomach acid, bile, and digestive enzymes. In addition, the microorganism must remain alive throughout its shelf life. On the other hand, non-digestible food compounds that can be metabolized by the microorganisms in the gut and increase the proliferation and activity of beneficial bacterial species in the microbiota are called prebiotics (Bindels et al., 2015). Most prebiotics consist of indigestible oligosaccharides. The most commonly used prebiotics are galacto-oligosaccharides, fructo-oligosaccharides, lactulose, and inulin. Compounds that contain both prebiotics and probiotics together are called synbiotics. Foods that contain live microorganisms are not considered ‘probiotics’, rather it has been proposed to consider these foods as foods containing live and active cultures. There are studies reporting that fermented milk and dairy products, which are among the leading examples of this food group, may be beneficial in glucose homeostasis, thus lessening the risk of type 2 diabetes mellitus, and

reducing the insulin resistance and systolic blood pressure (Tong et al., 2011; Wang et al., 2013).

The beneficial effects of probiotics have been tried to be explained by various mechanisms. Probiotics suppress the growth of potentially pathogenic bacteria and improve the intestinal barrier function. They interact with mucosal immunity and assist in the differentiation of regulatory T cells. On the other hand, probiotics regulate the immune system functions by affecting cytokines such as interleukin-8 (IL-8), interferon gamma, IL-10, transforming growth factor-beta (TGF-beta), and tumor necrosis factor (TNF). The effects of probiotics on immunity differ depending on the strain they contain. Probiotics also cause an increase in the turnover time of enterocytes. They have also been asserted to modulate the pain function in the bowels. Similar to commensal microorganisms, probiotics play a key role in many metabolic events such as deconjugation of bile acids, synthesis of vitamins, and production of short-chain fatty acids. However, these effects are not observed at the same rate in all probiotics, so the expected benefit from each probiotic is different (Maldonado Galdeano et al., 2019; Sanders et al., 2019; Sartor & Wu, 2017). The use of probiotics in gastrointestinal diseases has been increasing in recent years and is attracting more attention. In a study in which a detailed literature review on the use of probiotics in healthy individuals was conducted, it has been reported that the probiotic use in healthy individuals caused small and temporary changes in the microbiota. Again, in this study, it was stated that probiotics slightly reduced the frequency, symptoms, and duration of the common cold,

and that they had minimal or no effects on lipid profile, body mass index, and blood sugar levels (Khalesi et al., 2019). Probiotics have different genetic structures according to the microorganism strain they contain and they demonstrate different physiological activities. The health benefits obtained from one strain of probiotics cannot be generalized to all (Guarner et al., 2012).

3. THE USE OF PROBIOTICS IN GASTROINTESTINAL DISEASES

3.1. Irritable Bowel Syndrome

Diet and pharmacological treatment have an important role in the treatment of patients with irritable bowel syndrome (IBS). Removing gas-producing foods from the patients' diets may provide benefit in treatment. A diet low in monosaccharides, disaccharides, oligosaccharides, and polyols can reduce patients' complaints. In some selected patients, avoidance of lactose and gluten may add to the treatment. Particularly, in some of the diarrhea-dominant IBS patients, a response to the treatment can be obtained by changing the enteric bacterial composition with the administration of rifaximin (Chey, 2017; Ringel & Carroll, 2009). In IBS, adequate treatment may not be provided to all patients with diet and pharmacological treatments. Considering the pathogenesis of IBS, it has been suggested that replacing the commensal microbial diversity in the microbiota with probiotics may prove effective in the treatment. In a placebo-controlled study, when *Bifidobacterium bifidum* MIMBb75 probiotic strain was given to IBS patients for eight weeks, their symptoms

receded (Andresen et al., 2020). It has been reported that *Bifidobacterium infantis* 35624 probiotic is more effective than placebo when used at a dose of 1×10^8 CFU for four weeks (Whorwell et al., 2006). On the other hand, while *Lactobacillus plantarum* 299V probiotic was shown to be partially effective in a study, it was suggested in another study that it had no effect (Carroll et al., 2011; Sen et al., 2002). Although in numerous studies probiotics are thought to have potential benefits in relieving the symptoms of the IBS patients, it seems that these effects have not been fully proven (Moayyedi et al., 2010; Parker et al., 2018; Whelan & Quigley, 2013). The American Gastroenterological Association's (AGA) clinical guidelines do not recommend the use of probiotics in patients with IBS outside clinical studies (Su et al., 2020).

3.2. Constipation

Studies with the *Bifidobacterium lactis* DN-173010, *Lactobacillus reuteri DSM* 19738, and *E. coli* Nissle 1917 strains in patients with functional constipation have reported benefits in symptom relief (Yang et al., 2008; Riezzo et al., 2018; Chmielewska & Szajewska, 2010). In a meta-analysis, it was reported that the design and study results in functional constipation studies were heterogeneous and that probiotics were ineffective in functional constipation (Dimidi et al., 2014; Miller et al., 2017). Today, the use of probiotics in functional constipation is not recommended since their effects are not proven (Harris et al., 2019; Martínez-Martínez et al., 2017).

3.3. Antibiotic-Associated Diarrhea

With the increasingly widespread use of antibiotics, we encounter more and more antibiotic-associated diarrhea as a side effect. Diarrhea may develop by various mechanisms as a result of changes in the normal colonic mucosa after the use of antibiotics. Antibiotic-associated diarrhea occurs by various mechanisms, such as the osmotic effect of unabsorbed carbohydrates, insufficient deconjugation and degradation of bile salts by the reduction of bacteria, or infection by microorganisms such as *Clostridium perfringens* type A, *Staphylococcus aureus*, and *Salmonella enterica* (Olsen et al., 2001).

It has been suggested that probiotics may be effective in the prevention and treatment of antibiotic-associated diarrhea, as the condition is thought to occur with the changes in the colon microbiota. In studies with probiotics containing the *Saccharomyces boulardii* and *Lactobacillus* species, some reported that both probiotics reduced the frequency of antibiotic-associated diarrhea, whereas others contrarily stated they had no effect (Arvola et al., 1999; Lewis et al., 1998; Surawicz et al., 1989; Thomas et al., 2001). In a meta-analysis examining eight double-blind, randomized, placebo-controlled studies on probiotics containing the *Saccharomyces boulardii* and *Lactobacillus rhamnosus* GG strains, it was reported that probiotics containing these bacteria reduced the risk of antibiotic-associated diarrhea (D'Souza et al., 2002). The use of these probiotics benefits patients, especially those with a high risk of antibiotic-associated

diarrhea and who have had the disease before. It has also been reported that probiotics containing the combination of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* (Bio-K + CL1285) may be effective in preventing antibiotic-associated diarrhea, in addition to yogurts containing *Lactobacillus casei* DN114, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* (Quigley, 2021).

3.4. *Clostridioides Difficile* Infection

Pseudomembranous enterocolitis is an intestinal infection that can develop with toxin-producing the *Clostridioides difficile* strains in almost all patients after the use of an antibiotic. *Clostridioides difficile* infection (CDI) is becoming more common with the increase in the frequency of antibiotic use (Su et al., 2020). In a systematic review with meta-regression analysis, it was reported that the administration of probiotics close to the antibiotic dose reduced the risk of developing CDI (Shen et al., 2017). Another study conducted on patients with CDI demonstrated that there was no significant difference between the two patient groups in terms of CDI recurrence, when comparing patients who were given standard treatment and those who were given probiotics containing four strains in addition to standard treatment (Barker et al., 2017). It has been recounted that recurrence was less common in CDI patients who were treated with *Saccharomyces boulardii* probiotics (Surawicz et al., 2000). In a Cochrane analysis of four studies examining the benefits of probiotics in the treatment of CDI, no sufficient evidence to support the benefits

of probiotics in CDI was found (Pillai & Nelson, 2008). In the American College of Gastroenterology (ACG) clinical guidelines for the prevention, diagnosis, and treatment of CDI, the use of probiotics is recommended for both the prevention of the development of CDI in patients treated with antibiotics as primary prevention and the deterrence of relapse in patients with CDI as secondary prevention (Kelly et al., 2021). In a detailed technical analysis review, it was reported that the level of evidence for the effect of probiotics in the prevention or treatment of CDI during antibiotic use was low (Preidis et al., 2020). In AGA's guidelines, the addition of probiotics to the treatment is not recommended for patients with CDI, except during clinical researches. On the other hand, in patients with a high risk for the development of CDI and in selected adult and children patients using antibiotics, the use of one of the following probiotics; those containing the *S. boulardii* strain, those containing two strains as *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R, those containing three strains as *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus,* and *Bifidobacterium bifidum,* or those containing four strains as *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Bifidobacterium bifidum,* and *Streptococcus salivarius* subsp. *thermophilus* has been recommended in preventing CDI (Su et al., 2020).

3.5. Collagenous Colitis

Collagenous colitis is a subtype of microscopic colitis with diarrhea. It is characterized by an increase in the amount of subepithelial

lymphocytes and collagen in the colonic mucosa. The predominant symptom in collagenous colitis is diarrhea. In a study conducted with a low number of patients with *Escherichia coli* Nissle 1917, it was reported that probiotics containing the above strain may have a possible benefit (Tromm et al., 2004).

3.6. Celiac Disease

Celiac disease is an immune-mediated enteropathy, characterized by hypersensitivity to gluten in genetically predisposed individuals (Ludvigsson et al., 2013). It has been reported that the quantity of *Lactobacillus* and *Bifidobacterium* in the intestines are decreased in celiac patients (Lorenzo Pisarello et al., 2015). In a double-blind randomized controlled study, it was suggested that *Bifidobacterium infantis* could alleviate symptoms in untreated celiac patients (Smecuol et al., 2013). The evidence regarding the benefits of using probiotics in celiac disease is insufficient.

3.7. Radiation Enteritis

Enteritis secondary to radiation is a common complication that mostly causes diarrhea. Radiation-induced diarrhea may develop due to the occurrence of one or more of the following mechanisms, such as malabsorption of lactose and bile acids, changes in intestinal flora, deterioration in intestinal motility, or a deterioration in secretion, absorption, and immune functions of the intestinal system (Blanarova et al., 2009). In enteritis secondary to radiation, intestinal bacteria contribute to intestinal repair (Packey & Ciorba, 2010). In a study conducted with probiotics containing the *Lactobacillus acidophilus*

LAC-361 and *Bifidobacterium longum* BB-536 bacteria, it was reported that a standard dose could reduce the complaint of diarrhea due to radiation (Demers et al., 2014). In a meta-analysis including a randomized controlled trial, it was asserted that probiotics may be beneficial in preventing diarrhea during radiation therapy (Liu et al., 2017).

3.8. Lactose Intolerance

The *Lactobacillus acidophilus* DDS-1 strain has been reported to cause a decrease in diarrhea, vomiting, and cramp-like pain seen after lactose loading in patients with lactose intolerance (Pakdaman et al., 2016). This study suggests that the use of lactase-producing probiotics in patients with lactose intolerance has the potential of reducing symptoms.

3.9. Small Intestinal Bacterial Overgrowth

In a meta-analysis, it was reported that the use of probiotics did not cause a significant increase in the frequency of small intestinal bacterial overgrowth disease compared to non-users, and that although the use of probiotics in patients with small intestinal bacterial overgrowth reduced abdominal pain, there was no significant improvement regarding diarrhea (Zhong et al., 2017).

3.10. Crohn's Disease

In a study conducted on 35 patients with active Crohn's disease, it was reported that synbiotics therapy containing *Bifidobacterium longum* and Synergy 1 improved the clinical symptoms (Steed et al., 2010).

An overall evaluation of different studies suggest that the data do not support the use of probiotics in the induction and maintenance of remission in patients with Crohn's disease (Limketkai et al., 2020; Parker et al., 2018; Preidis et al., 2020). AGA recommends the use of probiotics for adults and children with Crohn's disease only in the context of a clinical research (Su et al., 2020).

3.11. Ulcerative Colitis

The addition of probiotics containing *E. coli* Nissle 1917 to the steroid treatment in the induction of remission of mild and moderately active ulcerative colitis has been reported to provide similar benefits as the addition of 5-aminosalicylic acid (5-ASA) to the treatment (Rembacken et al., 1999). In the maintenance of remission of mild and moderately active ulcerative colitis, it was found that the maintenance of remission was better in patients receiving *Bifidobacterium* compared to placebo, and that it can reduce relapse rates (Ishikawa et al., 2003). In studies conducted with the *E. coli* Nissle 1917 and *Lactobacillus rhamnosus* GG strains, the use of the strains did not improve relapse rates in mild and moderately active ulcerative colitis patients, whereas *Lactobacillus rhamnosus* GG was effective in prolonging the remission periods (Kruis et al., 2004; Zocco et al., 2006). In a small-scale study of 24 patients with mild to moderately active ulcerative colitis, it was suggested that there may be a potential benefit of adding *Saccharomyces boulardii* to 5-ASA (Guslandi et al., 2003). In a randomized controlled study using *Trichuris suis* eggs in patients with active ulcerative colitis, the group receiving *Trichuris*

suis treatment had significantly higher recovery rates compared to placebo (Summers et al., 2005). In studies conducted using the VSL#3 probiotic consisting of eight bacteria strains (*Lactobacillus acidophilus, L. plantarum, L. paracasei, L. bulgaricus, Bifidobacterium breve, B. longum, B. infantis,* and *Streptococcus thermophilus*), it was asserted that the probiotic could increase the remission while decreasing the disease activity in patients with mild and moderately active ulcerative colitis (Bibiloni et al., 2005; Huynh et al., 2009; Sood et al., 2009). Although studies have demonstrated that some probiotics show promise in the treatment of ulcerative colitis, many studies had a small population of patients and heterogeneous results. Therefore, due to the presence of risks associated with the use of probiotics in these patients and the lack of conclusive evidence for their benefits, there is no recommendation for the use of probiotics other than pouchitis in ulcerative colitis cases. In adults and children with ulcerative colitis, AGA does not recommend the use of probiotics outside clinical researches (Su et al., 2020).

3.12. Pouchitis

Restorative proctocolectomy and ileal pouch-anal anastomosis (IPAA) are common procedures performed on ulcerative colitis and familial adenomatous polyposis (FAP) patients. In patients with IPAA, idiopathic non-specific acute inflammation of the ileal pouch is called pouchitis. Pouchitis is the most common complication in patients with IPAA, with a prevalence of 16%-48% (Murrell et al., 2009). The condition is more common in patients with ulcerative colitis who

underwent IPAA. The cause of pouchitis is not entirely clear. It has been suggested that a decrease in the production of short-chain fatty acids and a change in the diversity of the microbiota cause the development of pouchitis (Sandborn et al., 1994). The condition is thought to be caused by an excessive immune response to altered intestinal bacteria in genetically predisposed individuals. It has been reported that the VSL#3 probiotic is effective in maintaining the remission in the treatment of recurrent and treatment-resistant pouchitis in chronic cases (Mimura et al., 2004). The use of the VSL#3 probiotic is recommended for initial maintenance therapy in patients with a relapse within the first four weeks after responding to antibiotic therapy or in patients with three or more relapses per year. In a study investigating the use of VSL#3 in remission maintenance, the recurrence rate after nine months was lower than that with placebo (Gionchetti et al., 2000). In a Cochrane systematic analysis, VSL#3 was found more effective than placebo in preventing the development of pouchitis and maintaining maintenance therapy in patients who have achieved remission with antibiotics (Holubar et al., 2010; Singh et al., 2015). The VSL#3 probiotic prevents the development of pouchitis and helps maintain remission (Magro et al., 2017).

3.13. Acute Infectious Gastroenteritis

Although acute infectious gastroenteritis is mostly self-limited, it causes labor loss and discomforting symptoms in patients. In a Cochrane analysis examining the studies in patients with acute infectious gastroenteritis, it was reported that *Lactobacillus*

rhamnosus ATCC 53103, *Enterococcus faecium* SF68, and *Saccharomyces boulardii* can reduce the frequency and duration of diarrhea (Allen et al., 2010).

3.14. Helicobacter Pylori Infection

Probiotics can cause an inhibitory effect on *Helicobacter pylori*. It has been suggested that adding probiotics to antibiotics for the treatment of *Helicobacter pylori* may increase the effectiveness of the treatment, decrease the side effects of the drugs, and allow the drugs to be tolerated more easily. *Saccharomyces boulardii* CNCM I-745 can reduce the side effects associated with *Helicobacter pylori* treatment (Quigley, 2021). In a meta-analysis evaluating randomized controlled studies, it was reported that the use of probiotics during the eradication treatment of *Helicobacter pylori* caused an increase in eradication success rates, and a decrease in drug-related side effects and in disease-related symptoms. However, it has also been stated that these results were heterogeneous and that they should be interpreted carefully (Lü et al., 2016).

3.15. Hepatic Encephalopathy

In hepatic encephalopathy, modulating the colonization of ammonia-producing bacteria in the intestinal microbiota is beneficial in the treatment. Lactulose therapy is used as a prebiotic in the treatment of hepatic encephalopathy. Lactulose exerts its effect by changing the intestinal pH, decreasing gastrointestinal transit time, and increasing fecal nitrogen excretion. It was reported that ammonia levels significantly lowered when probiotics were given to patients with

hepatic encephalopathy. In a meta-analysis, probiotics were found to be more effective than placebo in hepatic encephalopathy, but showed no benefit compared to lactulose (Dalal et al., 2017). In a randomized controlled study investigating the efficacy of probiotics in secondary prophylaxis of hepatic encephalopathy, patients were divided into three groups: those untreated, those given lactulose, and those given probiotics (VSL#3). The rate of development of recurrent hepatic encephalopathy was 57% in the untreated group, 27% in the lactulose group, and 34% in the VSL#3 probiotic group. The development of recurrent hepatic encephalopathy was significantly lower in patients who received lactulose and VSL#3 compared to those who did not receive any treatment, while there was no significant difference between patients receiving lactulose and VSL#3 treatments (Agrawal et al., 2012).

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CHAPTER 7

TREATMENT STRATEGIES AND DRUG REPURPOSING CANDIDATES IN COLORECTAL CANCER

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INTRODUCTION

In the estimates of GLOBOCAN (The Global Cancer Observatory) 2018, colorectal cancer (CRC) has been reported to be the third most commonly diagnosed cancer type (10.2% of total) in the world among both sexes (male & female). The death estimate was 551,269 people from CRC in the worldwide. CRC has been shown to be the second most common cause of death from cancer according to this information (Bray et al., 2018). Despite statistics, CRC frequently qualifies as preventable cancer because of developing from operable precursor adenomas that can be removed after detection with a screening colonoscopy.

There are various current procedures such as colonoscopy, fecal immunochemical occult blood tests (FIT), and fecal guaiac-based occult blood tests (FOBT) for the detection of CRC, however they have disadvantages or limitations in terms of cost and affecting mortality (Okugawa et al., 2014). New diagnostic/prognostic biomarkers and candidate repurposed drugs effective to change the course of the disease are needed to deal with this highly fatal type of cancer.

Since the information obtained from experimental studies are stored globally in publicly accessible databases such as ArrayExpress (Parkinson et al., 2007) and National Center for Biotechnology Information – Gene Expression Omnibus (NCBI-GEO) (Barrett et al., 2013), diseases can be investigated at the macroscopic level and new

methods for diagnosis and treatment targets can be developed in a cost-effective and time-saving manner. In brief, this public accessibility removes various limitations such as time, location and clinical trial authorizations for computational biology.

Systems biology considers the events in living organisms on the system's levels whereas it helps us to explain physiological, molecular, and genetic events and total relations among these events. The computational techniques are used to analyse raw data from experiments or clinical trials through processing the data and making them suitable for integration and further analysis. If the limited experimental data are integrated through a system-oriented computational approach, it gets easier to make sense of the complex biological systems (Azuaje, 2010).

In recent years biomarker discovery came into prominence on prognosis and diagnosis of diseases as well as CRC. In this direction, cancer researchers especially focused on the identification of robust and reproducible molecular markers for personalised medicine. The systems biology perspective provides great conveniences during the discovery of new biomarkers.

Drug repurposing in systems pharmacology, which is the application area of systems biology, can be considered among the time and cost-saving methods and applications employed to prevent or treat the diseases. In these approaches, the existing drugs that have been proven to be reliable in preclinical models and humans, and whose indications

in the treatment of a particular disease have already been acknowledged are investigated for the treatment of another disease in which the indications have not been previously revealed (Pushpakom et al., 2018). While the period between the discovery of a drug and clinical trials takes approximately 9 years, in case the drug is repurposed, this period decreases to 3 or 4 years (Ashburn & Thor, 2004).

Several drug candidates have been represented previously by researchers for the diagnosis and treatment of CRC, by integrated perspectives. In a study, Clatom (Tolfenamic acid), a drug that reduces the pain caused by migraine, was shown as a repurposed candidate for CRC treatment. The researchers conducted transcriptome profiling and statistical analyses using colorectal adenomas from patients, and rat colon polyps selected as colon preclinical model. Then, common differentially expressed genes (DEGs) were determined between colorectal adenomas and colon polyps. In the colonic preclinical model, clinically relevant doses of clatom were shown to suppress carcinogenesis, particularly by affecting four oncogene candidates (Aldh1a3, Mmp7, Nppb, and S100a9) (Ertem et al., 2017).

Etacrynic acid, 6-azathymine, GW-8510, ginkgolide A and some other drugs, were presented as repurposed drug candidates for CRC through a systems cancer treatment approach. This integrated analytical framework was named Functional Module Connectivity Map (Chung et al., 2014).

In another study using a computational approach, microarray data sets belonged to CRC patients were obtained from the GEO database, DEGs were determined, and 11 small molecules that could reverse the expressions of dysregulated genes were presented through the Connectivity map (Lamb, 2007). These small molecules were asiaticoside, DL-thiorphan, doxylamine, ellipticine, heliotrine, methocarbamol, methylprednisolone, novobiocin, piperlongumine, ursodeoxycholic acid and, vorinostat (Zheng et al., 2020).

Currently available diagnostic biomarkers for early detection or treatment protocols specific to CRC are not sufficient to overcome this worldwide burden of disease. Novel cost-effective and time-saver strategies are needed for the prevention of disease progression and, treatment.

1. ENVIROMENTAL RISK FACTORS OF COLORECTAL CANCER (CRC)

Several endogenous and environmental stress factors are reported to be related to the carcinogenesis of CRC. These stress factors may lead to activating several biological pathways that are involved in disease progression (Diao et al., 2016).

Although the annual age-standardized incidence rate of CRC is below four in a few less developed countries, it was reported that the rate exceeds forty in developed and more industrialized countries. In these countries, CRC still appears to be one of the major health problems. There was a tenfold variation in global CRC rates when the same

generation migrated from low-risk regions to high-risk regions. These data underscore a strong environmental effect on the pathogenesis of CRC (Dunn, 1975; Parkin, 2004). Although data on the risk factors are limited in many regions around the world, the rapid increase in the CRC rate in newly developed or developing countries under an economic change was associated with many reasons such as rapid population growth, the increase in life expectancy, smoking, physical inactivity and ready-to-eat food consumption characterized with western-style eating habits that lead to obesity (Center et al., 2010).

Obesity, which more than a billion people suffer from worldwide, is a condition of overweight that identifiable when the body mass index rises above a certain rate. In many epidemiologic studies, obesity has been reported as a strong risk factor for CRC and it also has been stated as a remarkable risk factor for many types of diseases, including some other types of cancer (Matsuo et al., 2012).

In a comprehensive epidemiologic study conducted among anatomic sites, the relative risk of proximal colon cancer has been identified statistically significantly increased with diabetes mellitus (Limburg et al., 2005). In another study, researchers showed that diets rich in simple carbohydrates, which increase plasma glucose levels, increase the risk of colon cancer compared to diets containing complex carbohydrates (Slattery et al., 1997).

The metabolic syndrome characterized by the combination of glucose intolerance, dyslipidemia, obesity and high blood pressure

(hypertension) has been reported as a metabolic disorder. The incidence of metabolic syndrome and colon cancer, which have overlapping risk factors, has also increased in parallel with the dramatic increase in obesity in developing countries (Chiu et al., 2007).

In a study, long-term and high consumption of processed red meats has been notified to increase the developing risk of CRC in the distal part of the large intestine. In the same study, it was shown that long-term poultry and fish consumption were inversely related to the risk of both proximal and distal colon cancer (Genkinger & Koushik, 2007). Looking at GLOBOCAN data, it could be observed that there is a harmony between CRC and dietary data containing animal fat and meat ratio in foods consumed in many parts of the world (Faraz Bishehsari et al., 2014).

Alcohol has been reported to be a sufficient causal factor for CRC with sufficient proven data available, by the International Agency for Research on Cancer (IARC) (Pelucchi et al., 2011). The increase in the risk of CRC with alcohol intake can be explained as follows: when alcohol enters the colon, it is microbially metabolized to acetaldehyde, which degrades folate in vivo, which has an important role in essential biological processes such as DNA repair and synthesis (Homann et al., 2000). In the case of folate deficiency, when cytosine methylation alters in DNA by intracellular S-adenosylmethionine decreasing, this situation may lead to improper activation of proto-oncogenes and thus malignant transformation may be induced. Uracil misincorporation

into DNA, instability in DNA precursors, and chromosome breakage, are also possible risks leading to instabilities that can occur in the case of folate deficiency (Duthie, 1999).

Many studies have shown that tobacco exposure increases the CRC development risk (Amitay et al., 2020; Cross et al., 2014). A significant association between cigarette smoking and CRC has been shown in terms of both incidence and mortality, in a comprehensive study including many observational studies and conducting a meta-analysis (Botteri et al., 2008). In another study, researchers revealed that O-cresol sulfate, hydroxycotinine, and serum cotinine, are significant biomarkers that represent individual variation in tobacco metabolism, and tobacco consumption habits. Hydroxycotine in particular has been found to be significantly associated with CRC malignancy (Cross et al., 2014).

Age-related studies also confirm the unfavourable effects of increased environmental risk factors on CRC due to westernization. In a study investigating epidemiologic patterns, it was found that the rate of cancer in younger individuals was higher than in older individuals. Young people here were more closely related to environmental factors such as diet and lifestyle that have changed in recent years, so the rate of CRC was higher in these individuals than in the elderly (Ansari et al., 2006).

2. GENETIC AND EPIGENETIC ALTERATIONS REPORTED FOR COLORECTAL CANCER (CRC)

To define cancer effectively, which is a somatic evolutionary process, it is necessary to identify the genetic and epigenetic changes underlying its mechanisms (Bodmer, 2008). Developing new approaches to cancer prevention and treatment will be possible by understanding the function of these changes. Along with the environmental factors mentioned above, CRC has an important genetic basis. CRC is a good model for both studying somatic evolution of epithelial cancers and susceptibility to the disease since the different stages of disease are more easily reachable than many other cancer types and inherited susceptibilities are well-defined in CRC.

CRC could be inevitable at the age of 20 or 30 in individuals affected by familial adenomatous polyposis (FAP), which is reported to be most well-known familial syndrome, furthermore, in individuals with FAP, the number of precancerous growths such as polyps or adenomas could reach a few hundred to more than a thousand (Bodmer, 2008).

Mutation spectra, which can show in which region (coding or non-coding) of the genome the mutation has occurred, or what types of mutations have occurred at what rate, could have different appearances because they reflect different genetic and environmental factors (Zhunussova et al., 2019). Compared to a table that gives the

mutation rate alone, the mutation spectrum is important in terms of being able to show in detail and understand what is going on in a genome. In one study, researchers detected genetic changes in major genes such as TP53 and KRAS in CRC samples from high-risk regions but found that the spectra of the mutations were quite different. Researchers have implicated these differences with different environmental exposures (Bishehsari et al., 2006).

In another study examining gene mutation frequencies and DNA methylation patterns in CRC the cases belonging Middle Eastern countries including Turkey, Egypt, and Jordan were evaluated. The microsatellite instability pathway, TP53 and KRAS gene status, and CpG island methylator phenotype pathway characterized by proper methylation of gene promoters that silences the transcription of genes were evaluated. The lowest methylation frequency was seen in CRC samples from Egypt. By multinomial logistic regression analysis, methylation including the MINT31 locus and the CDKN2A tumor suppressor gene was found more frequently in Jordan CRC samples. The TP53 overexpression was more common in both Jordanian and Turkish CRC than in Egyptian cases, but the KRAS proto-oncogene was mutated more frequently in CRC samples from Turkey. It was observed that the findings reported for Western cases were mostly similar to those in Turkish CRC (Chan et al., 2005). It can be stated that these regional differences probably reflect different environmental exposures, have different mutation frequencies, and different gene methylation patterns indicating molecular pathogenesis.

CRC arising through a series of well-characterized histopathological changes is associated with specific genetic changes in tumor suppressor genes and responsible oncogenes. Various genes that are mutated in sporadic CRC or whose expression levels change as a result of mutation effects and induce carcinogenesis have been identified. For example, APC (Adenomatous Polyposis Coli), CDH1 (E-cadherin), and CTNNB1 (β-catenin) are known to affect the Wnt pathway. The TP53 gene involved in apoptosis, CDKN2A gene alternate protein products p14ARF and p16INK4a involved in cell cycle checkpoint, mismatch repair genes such as hMSH6, hMLH1, and hMSH2, transcription factors (TFs) such as SMAD4 and their receptors or signalling associated genes (KRAS, TGFbetaIIR), and antitumor immune response associated genes such as HLA Class I and beta2m, were also reported for sporadic CRC (Bodmer, 2008).

The Wnt signaling pathway, which is known to cause cancer and many diseases as a result of its deregulation (MacDonald et al., 2009), has an essential function in the developmental process of the cell, cell proliferation, maintaining the polarity of the cell, and tissue haemostasis (Logan & Nusse, 2004). It has been found that in CRC cases, Wnt signaling pathways change over 90% and this difference involves the inactivation of the APC gene and CTNNB1 activation caused by mutations in approximately 80% of cases (Muzny et al., 2012). A truncated protein is formed as a result of APC mutations in a region of open reading frame called MCR (mutation cluster region). The changes in question at APC have been characterized by the

inactivation of Axin binding sites (SAMP), C-terminal basic region, nuclear localization signals, and multiple β-catenin binding regions (20R) (Parker & Neufeld, 2020). The lack of β-catenin reaching the nucleus as a result of continuous elimination leads to suppression of Wnt target genes by DNA-bound TCF (T cell factor) /LEF (lymphoid enhancer factor) family proteins (MacDonald et al., 2009).

The tumor suppressor p53 protein known to be mutated abundantly in CRC (Baker et al., 1990), is a TF that triggers apoptosis and affects the normal developmental process of the cell by stopping the cell cycle under cellular stress or by inducing aging (Li et al., 2015). There are several clinical trials and laboratory evidence supporting that restoration or reactivation of p53 by various small molecule inhibitors or medicines can induce apoptotic pathways such as death-receptor-induced or mitochondrial (Tan et al., 2005; Zhou et al., 2008).

3. SCREENING METHODS OF COLORECTAL CANCER (CRC)

Screening is matter in terms of preventing the mortality, morbidity, and excessive cost of cancer treatment through detecting significant lesions that tend to become cancerous or detecting early-stage cancer that tend to spread beyond the intestinal wall. Screening tests for CRC are divided into two groups: indirect tests that investigate the presence of colorectal neoplasm markers in the stool, and direct tests providing to visualize the neoplasm arising in the large intestine. The most common screening tests currently used worldwide are biomarker

research-based tests including FOBT and FIT, and visualization tests including FS (flexible sigmoidoscopy) and TC (total colonoscopy) (Stracci et al., 2014).

In addition, there are commercial tests on the market that estimate the probability of cancer recurrence by detecting gene expression signatures after resection of cancerous tissue. These multigene-expression tests help to decide whether systemic treatment (adjuvant theraphy) is required to eliminate the remaining cells from the surgically resected tumor to reduce the risk of cancer recurrence (Sawyers, 2008).

4. BIOMARKERS IN COLORECTAL CANCER (CRC)

Biomarkers that provide clues to determine the type of cancer arising in a patient or detect cancer recurrence are called diagnostic biomarkers. Diagnostic tests detecting diagnostic biomarkers can be used with standard imaging techniques to increase the effectiveness of CRC screening (Newton et al., 2012). Prognostic biomarkers that give clues about how the natural course of any cancer is progressing can guide how to distinguish good outcome tumors from those with poor outcomes, and what type of and how aggressive treatment should be followed (Sawyers, 2008).

It is stated that carcinoembryonic antigen (CAE), a complex glycoprotein whose production is almost negligible in healthy adult cells, is produced in approximately 90% of CRCs and enhances tumor malignancy (Goldstein & Mitchell, 2005). The increase in the level of

CEA appears to be a negative prognosis factor in resectable CRC (Locker et al., 2006). Known as the most common blood-based CRC biomarker, the increase in levels of CEA has been reported not only specific to CRC but may also indicate the presence of liver disease, pancreatitis, inflammatory bowel disease, or some other malignancies (Hauptman & Glavač, 2017). The fact that CEA is a biomarker often used in practice to screen for CRC recurrence (Locker et al., 2006) may be attributed to the aforementioned unspecificities.

The aberrant methylated DNAs, which can be found in the stool or circulation, are among the biomarkers investigated in the diagnosis or prognosis of CRC. In a study, methylation of SEPT9 was investigated as a biomarker for its sensitivity and specificity using a blood based screening test in CRC (Warren et al., 2011). Collected plasma samples have been categorized in 3 cohorts as samples from untreated CRC patients (50 samples), control samples (94 control samples), and samples from asymptomatic patients undergoing colonoscopy (300 samples). In this study, concentration of methylated SEPT9 in the patients' plasmas was measured and the specificity and sensitivity levels were determined by statistical analysis. CRC was successfully detected in patients at all stages and SEPT9 methylation had a specificity of 88% and an overall sensitivity of 90% for CRC. The test, which has high specificity for methylated SEPT9 can also be used for colorectal locations of CRC, makes it an alternative that can be offered to people at average risk who cannot or do not want to have a colonoscopy.

In another study, a stool-based test named multi-target stool DNA (MT-sDNA) investigating BMP3 and NDRG4 promoter methylation, KRAS mutations, and haemoglobin has been compared with the FIT. Stool samples from CRC patients with non-advanced adenomas, advanced precancerous lesions (advanced adenoma and advanced serrated polyps), and control samples (negative findings) were obtained to compare the sensitivity and specificity of MT-sDNA test with that of FIT. The MT-sDNA test was found to be more successful in detecting precancerous lesions with 46%, which was higher than that of FIT (27%). It was seen that comparing the individuals with non-advanced or negative findings (controls), the MT-sDNA test exhibited 89% specificity, while the specificity for FIT was 93%. In detection of high-risk advanced adenomas, both the MT-sDNA test and FIT showed no significant sensitivity. According to these results, in an average-risk screening population, the MT-sDNA test has a higher sensitivity compared to FIT in detecting only advanced precancerous lesions (Bosch et al., 2019).

There are various clinical and preclinical studies reporting the vascular endothelial growth factor (VEGF) is the predominant angiogenic factor inducing growth of new capillary blood vessels in CRC. The VEGF increase has been associated with distant metastasis and advanced lymph node status in CRC, and it has been notified that advanced increase in VEGF expression indicates a poor prognosis (Bendardaf et al., 2008).

Qi et al. conducted a study integrating computational bioinformatics analysis and experimental validation to explore the key genes and molecular pathways which underlie CRC metastasis. Using the datasets from NCBI-GEO, DEGs have been identified through statistical analyses. A protein-protein interaction network has been constructed and this way the top ten hub genes have been identified. After subnetwork and pathway enrichment analyses, a reverse transcription-quantitative polymerase chain reaction assay has been conducted to confirm the expression of 5 candidate genes associated with metastasis. Researchers selected 5 hub genes (EGFR, HRAS, Wnt5a, Akt1, and CDKN1a) with the highest interactional degree of all genes as the most prominent potential metastasis-associated DEGs. The results of the validation experiment using clinical tumor samples were overlapping with the results of the 5 bioinformatically selected DEGs. It was found that the mRNA expression levels of up-regulated DEGs (EGFR, HRAS, and Akt1) were increased and the expression levels of down-regulated DEGs were (Wnt5a and CDKN1a) decreased in metastatic samples. Researchers concluded that these 5 genes could be key DEGs in the metastasis of CRC, based on previous studies and the validation results in this study (Qi et al., 2018).

In a study circulating miRNAs were investigated by network-based analysis in terms of affecting to CRC prognosis. Researchers conducted miRNA profiling and then constructed a miRNA-mediated gene regulatory network. They obtained a gene regulatory network from the ORTI database compiling the mammalian TFs and their

associated TGs (target gene) from publicly available databases including TF−TG interactions such as TRED, TRRD, HTRI, TFactS, PAZAR, and NFI-Regulome, and the literature. A class of optimization algorithms named Evolutionary Algorithms (EAs) has been used to overcome the optimization problem. Researchers developed an innovative multi-objective optimization-based computational framework to identify miRNA biomarkers using both the miRNA expression profile and information from the miRNA-mediated regulatory network previously constructed by them. By conducting the bioinformatics analysis using 'HTqPCR' R package, 11 plasma miRNAs namely hsa-let-7a, hsa-miR-106a, hsa-miR-185, hsa-miR-21, hsa-miR-217, hsa-miR-25, hsa-miR-483-5p, hsa-miR-30a-5p, hsa-miR-431, hsa-miR-615-5p, hsa-miR-892a1 have been revealed as prognostic signature. Researchers reported that these prognostic signatures overlapping with previous findings on miRNA prognostic markers detected from plasma or tumors of CRC patients (Vafaee et al., 2018).

In another study aimed to explore key genes and their functions that have an important role in the progression of CRC researchers were obtained the GSE4107 and GSE8671 microarray data sets from the GEO database. Then, Limma package of R has been used for identifying DEGs from each data set. Thirty two genes have been revealed as the common DEGs in both datasets. Biological processes enrichment analysis and meta-analysis of DEGs have been conducted through the DAVID (Database for Annotation, Visualization and

Integrated Discovery) tool. Several significantly enriched GO (Gene Ontology) terms related to response to wounding, steroid hormone stimulus, drug, and inflammatory response etc. were identified. EPH receptor A3 (EPHA3), Chemokine (C-C motif) ligand 21 (CCL21), IL8, chemokine (C-X-C motif) ligand 13 (CXCL13), and CR2 (CD21) have been found as DEGs related to the inflammatory response. Fatty acid-binding protein 4, butyrylcholinesterase (BChE), UDP glucuronosyltransferase 1 family, polypeptide A6 (UGT1A6), member 2 (ABCG2), adipocyte (FABP4) and ATP-binding cassette, sub-family G (WHITE) have been found as DEGs related to the drug response. Angiopoietin-like 1 (ANGPTL1), chordinlike 1 (CHRDL1), EPHA3, and gremlin 2 (GREM2) have been found as DEGs involved in enzyme-linked receptor protein signalling pathway (Liu et al., 2013).

Liang et al. used the GSE21815 dataset from the GEO database to explore the key genes and pathways in CRC. There were 141 samples, including 132 CRC and 9 normal colon epitheliums. The data have been categorized through hierarchical clustering analysis. Three thousand five hundred DEGs have been identified by t-test; of which, 1370 were found up-regulated and 2130 were found to be down-regulated. All DEGs have been uploaded to the DAVID tool for the purpose of identify overrepresented GO categories and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. The GO term analysis showed that up-regulated DEGs were mainly involved in cell cycle, regulation of cell proliferation, and cell apoptosis

whereas down-regulated DEGs were involved in immune response, intracellular signalling cascade, defence response, and positive regulation of immune system process. The enriched KEGG pathways of up-regulated DEGs included cell cycle, ECM-receptor interaction, p53 signalling pathway, and ECM-receptor interaction. Down-regulated DEGs were related to the calcium signalling pathway. Researchers constructed the PPI network with DEGs and revealed the top degree hub genes: GNG2, AGT, SAA1, ADCY5, LPAR1, NMU, IL8, CXCL12, GNAI1, and CCR2. Among these genes, the highest node degree belonged to GNG2 was found as 60. The Molecular Complex Detection (MCODE) has been employed to screen the modules of the PPI network in Cytoscape. The top 3 significant modules have been selected, and the functional annotations of the genes involved in the modules have been analysed. Enrichment analysis showed that the genes in modules 1–3 were mainly associated with G protein-coupled receptors (GPCR) signalling pathway, the gastrin-CREB signalling pathway via PKC and MAPK, and extracellular matrix organization (Liang et al., 2016).

5. THE DRUGS USED FOR TREATMENT OF COLORECTAL CANCER (CRC)

Currently, there are various available drugs for the systemic treatment of CRC. These drugs or drug combinations administered according to the locations of the malignancy are presented in Table 1.

Table 1: FDA Approved Drugs For CRC. The Data Was Accessed From The Web Page Of National Cancer Instutute (https://www.cancer.gov/about-cancer/treatment/drugs/colorectal)

Drugs Approved for Colon Cancer	Avastin (Bevacizumab) Bevacizumab Camptosar (Irinotecan Hydrochloride) Capecitabine Cetuximab Cyramza (Ramucirumab) Eloxatin (Oxaliplatin) Erbitux (Cetuximab) 5-FU (Fluorouracil Injection) Fluorouracil Injection Ipilimumab Irinotecan Hydrochloride Keytruda (Pembrolizumab) Leucovorin Calcium Lonsurf (Trifluridine and Tipiracil Hydrochloride) Mvasi (Bevacizumab) Nivolumab Opdivo (Nivolumab) Oxaliplatin Panitumumab Pembrolizumab Ramucirumab Regorafenib Stivarga (Regorafenib) Trifluridine and Tipiracil Hydrochloride Vectibix (Panitumumab) Xeloda (Capecitabine) Yervoy (Ipilimumab) Zaltrap (Ziv-Aflibercept) Zirabev (Bevacizumab) Ziv-Aflibercept
Drug Combinations Used in Colon Cancer	CAPOX FOLFIRI FOLFIRI-BEVACIZUMAB FOLFIRI-CETUXIMAB FOLFOX FU-LV XELIRI XELOX
Drugs Approved for Rectal Cancer	Avastin (Bevacizumab) Bevacizumab Camptosar (Irinotecan Hydrochloride)

	Capecitabine Cetuximab Cyramza (Ramucirumab) Eloxatin (Oxaliplatin) Erbitux (Cetuximab) 5-FU (Fluorouracil Injection) Fluorouracil Injection Ipilimumab Irinotecan Hydrochloride Keytruda (Pembrolizumab) Leucovorin Calcium Lonsurf (Trifluridine and Tipiracil Hydrochloride) Mvasi (Bevacizumab) Nivolumab Opdivo (Nivolumab) Oxaliplatin Panitumumab Pembrolizumab Ramucirumab Regorafenib Stivarga (Regorafenib) Trifluridine and Tipiracil Hydrochloride Vectibix (Panitumumab) Xeloda (Capecitabine) Yervoy (Ipilimumab) Zaltrap (Ziv-Aflibercept) Zirabev (Bevacizumab) Ziv-Aflibercept
Drug Combinations Used in Rectal Cancer	CAPOX FOLFIRI FOLFIRI-BEVACIZUMAB FOLFIRI-CETUXIMAB FOLFOX FU-LV XELIRI XELOX
Drugs Approved for Gastroenteropancreatic Neuroendocrine Tumors	Afinitor (Everolimus) Everolimus Lanreotide Acetate Somatuline Depot (Lanreotide Acetate)

6. REPURPOSED DRUG CANDIDATES FOR COLORECTAL CANCER (CRC)

In recent years, drug repurposing has become an alternative method in overcoming different diseases due to its time-saver and cost-effective advantages over traditional approaches in drug design. Repurposing of an existing approved or investigational drug for a different indication is possible with extensive studies based on experimental or computational science (Nowak-Sliwinska et al., 2019). Various repurposed drugs or small molecules have been presented for improving survival or reversing the poor course of the disease in CRC by researchers.

Produced as a recombinant fusion protein, Aflibercept contains components that can bind VEGF from the extracellular domains of certain VEGF receptors (receptors 1 and 2) located in the Fc fragment of human immunoglobulin G. This drug is an intravitreal vascular endothelial growth factor (VEGF) inhibitor and its common use was known in treatment of Age-Related Macular Degeneration (AMD) (Lim et al., 2012), diabetic macular edema (Brown et al., 2015), and diabetic retinopathy (Ross et al., 2016). Interestingly, aflibercept is also one of the repurposed drugs for metastatic CRC clinically shown to contribute significantly to survival. In a study conducted with metastatic CRC patients who were previously received the oxaliplatin and bevacizumab in their treatment process, researchers have shown that the patient survival has been improved by adding Aflibercept to a

chemotherapy regimen called FOLFIRI, which consists of fluorouracil, leucovorin, and irinotecan (Van Cutsem et al., 2012).

Brigatinib, which is a tyrosine kinase inhibitor, was shown to be exhibit in vitro activity against epidermal growth factor receptor (EGFR) deletion, ROS1, FLT-3, insulin-like growth factor-1 receptor (IGF-1R), anaplastic lymphoma kinase (ALK), and several point mutations. It has been firstly approved as an effective small molecule inhibitor in treatment regimen of the patients with ALK-positive metastatic non-small cell lung cancer (Markham, 2017). Furthermore, brigatinib is an anti-cancer therapeutic that has been proven to contribute effectively to the induction of apoptosis by creating endoplasmic reticulum (ER) stress in CRC, which is an ALK-negative cancer type. By combining brigatinib with autophagy inhibitors, the researchers demonstrated that its anti-carcinogenic effect was increased independently of ALK and it was a potent repurposing candidate for CRC in vivo and in vitro (Zhang et al., 2019).

Celecoxib, one of the small molecules repurposed for FAP, has been reported to be a nonsteroidal anti-inflammatory drug and selectively inhibit cyclooxygenase-2 (COX-2). This drug with the brand name Celebrex is commonly prescribed for treatment of various diseases such as Rheumatoid Arthritis (RA), Osteoarthritis, Juvenile RA in patients 2 years and older, Acute Pain, Ankylosing Spondylitis, and Primary Dysmenorrhea (FDA US-Food and Drug Administration, 2014). In an in vivo study investigating the effects of celecoxib in patients with FAP, when patients were given 400 mg or 100 mg of

celecoxib twice daily for 6 months, a 28 % reduction in polyps was observed in patients who received the 400 mg dose after 6 months. With this significant reduction, celecoxib was presented as an effective repurposing candidate for FAP (Searle et al., 2000).

Doxycycline, a tetracycline antibiotic, is used to treat a variety of infectious agents, including Streptococcus pneumonia, Staphylococcus aureus, E. coli, and P. multocida (Cunha et al., 2000). In a study focusing on the contribution of matrix metalloproteinases (MMPs) and COX enzymes to the metastatic process in CRC, the effects of doxycycline, an MMP inhibitor, and NS-398, a COX-2 inhibitor, alone or in combination, on CRC cell lines were investigated. The combined therapy of Doxycycline and NS-398 was observed to have a pronounced antiproliferative and anti-invasive effect on CRC cell lines compared to single therapy with these drugs. Doxycycline in the combined treatment regimen, whose chemoprevention efficiency was proven by the findings obtained in this in vitro study, was proposed to be a repurposing candidate for the treatment of CRC (Onoda et al., 2004).

Etodolac, known as a non-steroidal anti-inflammatory drug, representing analgesic activity, inhibits prostaglandin biosynthesis. It has been approved and used for the treatment of pain, osteoarthritis, and inflammation associated with various forms of arthritis (Brocks & Jamali, 1994). Etodolac has been shown to proportionally decrease matrix metalloproteinase (MMP)-9 and COX-2 mRNA activities in

mice injected with a colorectal cell line, thereby preventing liver metastasis of CRC cells (Ishizaki et al., 2006).

Another repurposing candidate whose effect has been investigated in colorectal tumors is propranolol, which is widely prescribed to treat the hypertension (Zacharias, 1969) and infantile hemangiomas (Léauté-Labrèze et al., 2015). This drug is a non-selective β-adrenergic receptor blocking agent that has been shown to suppress tumor growth in various solid tumors in preclinical models. The therapeutic effect of propranolol for CRC was investigated with the CT26WT colon carcinoma cell line engrafted into BALB/C mice (Liao et al., 2020). In addition, in the same study, the effect of propranolol treatment was compared to patients with CRC who had not been treated before and who needed surgical resection, by administering propranolol 1 week before the operation or not using propranolol. In CT26WT tumors treated with propranolol, down-regulation of p-AKT, p-ERK, and p-MEK were observed. Significant increases in GzmB, IFN-γ, and T-bet expressions were also observed in the treated group with increased CD8+ T cells. In addition, tumor growth of the propranolol-treated mice was observed to be slower than that of the untreated mice. It was observed that p-ERK expression decreased and CD8+ T cells significantly increased in surgical patients receiving propranolol treatment. The suppression of CRC tumor growth by propranolol was revealed in these both preclinical and clinical studies.

In a study, based on the knowledge that high expression of MDR1 (Multidrug Resistance 1 also known as P-glycoprotein) protein, which is formed as a result of the expression of the ABCB1 gene, causes drug resistance in CRC, drug repurposing was carried out by establishing co-expression networks of various sizes around the ABCB1 gene. Using HT29 colorectal cell line expression data, differential co-expression genes were identified and candidate drugs that could reverse the expression of these genes were proposed. Some candidate drugs that were found to be important in reversing the effect caused by ABCB1 in the study are as follows: importazole, brazilin, Ro 28-1675, NCGC00181381-01, and PD 407824 (Beklen et al., 2020).

The effect of fluspirilene, a depot antipsychotic drug approved for the treatment of schizophrenia, on various cancers including CRC (Patil et al., 2015), glioblastoma (Dong et al., 2017), hepatocellular carcinoma (Shi et al., 2015), has been investigated. In a combined computational and experimental study, the researchers proposed fluspirilene as a potential p53-MDM2 inhibitor, where they demonstrated efficacy on inhibition of tumor growth in HCT116 colon cancer cell lines (Patil et al., 2015).

CONCLUSION

Integrated and comprehensive systems biology approaches are essential in terms of being approaches that can reveal the possible causes and mechanisms of cancer as well as treatment candidates. Making sense of transcriptome-based big data with biostatistical

calculations contributes to revealing the mechanisms underlying the disease pathogenesis, revealing the clinically actionable genes, and identifying new actions that can be taken for the treatment of the disease.

Since the design of a drug for disease requires long processes and heavy costs, the importance of drug repurposing, which can create treatment options for diseases, is well understood, especially during the COVID-19 pandemic. Considering these important benefits of drug repositioning, we proposed drug candidates that could be a treatment option for CRC, which still suffers from people all over the world, with an integrated approach that brings together many disciplines.

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CHAPTER 8

THE ROLE OF CELL PROLIFERATION IN THE CASE OF CHEMOTHERAPY RESISTANCE ON LEUKEMIA

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INTRODUCTION

Chemotherapy means the use of a drug (such as aspirin or penicillin) to treat a disease, however by most people, the term refers to drugs used to treat cancer. Chemotherapy is used to treat various types of cancer. More than 100 kinds of chemotherapy drugs are currently used, either alone or in combination with other drugs or therapies. These drugs vary widely in their chemical composition, route of administration, use in the treatment of certain cancers, and their side effects (Gascoigne *et al*., 2009).

Cell resistance can be caused by a decrease of drug metabolism to its active form, increase of drug inactivation, increase of cell defense mechanisms, changes in molecular targets, changes in cell death regulators. Many drugs are used as chemotherapy regimens for leukemia, and each drug has its own resistance mechanism, so there is no single mechanism that is responsible for clinical resistance (Su *et al*., 2011).

In principle, the clinical outcome of chemotherapy is determined by 2 factors: first, whether the drug can reach the leukemia cells and second, whether the drug which reach the cells can certainly kill the cells. The second factor concerns drug resistance at the cellular level. In addition, genetic heterogeneity among patients is also associated with clinical outcomes. Beside these two factors, other factors also determine the clinical outcome after chemotherapy. If the leukemic cells are resistant to chemotherapy, either because the drugs cannot reach them or because the cells are resistant to the drug, then it will

cause clinical problems due to these leukemia cells have the potential to grow back (Kaspers, 1993).

There are various chemotherapy drugs in leukemia with various mechanisms of action including the induction of apoptosis and inhibition of cell proliferation. This chapter will present how chemotherapy drugs act in general and how the mechanism of chemotherapy resistance through the way on inhibition of cell proliferation and also examination.of cell proliferation itself.

HOW DOES CHEMOTHERAPY DRUG ACT ?

There are three targets of anti-cancer treatment, as follows: 1) damage the DNA of cancer-affected cells; 2) inhibiting the synthesis of new DNA strand to stop cell replication because if the cell replicates, it means giving a chance to tumor to grow; 3) inhibiting mitosis or cell division into 2 new cells, thereby stopping the journey of cancer cells. Drugs that interfere with the course of mitosis, called anti-mitotic agents, are widely used for the treatment of cancer, including *taxanes* such as taxol which is widely used for the treatment of breast and ovarian cancer, and vinca alkaloids such as vincristine, which is often used in combination therapy for the treatment of hematological malignancies. Vinca alkaloids, isolated from *Catharanthus roseus (Madagascar periwinkle)* interact with b-tubulin in a region adjacent to the GTP-binding site known as the vinca domain (Gascoigne, 2009).

Based on their site and mode of action, chemotherapeutic drugs are classified into 3 main groups: antimetabolites, genotoxic agents, and inhibitors of the mitotic spindle.

1. ANTIMETABOLITES

The examples of drugs in this class include folate antagonists, pyrimidine, and purine. This class of drugs works as anti-neoplasmic because its structure and function are similar to natural metabolites that play a role in the synthesis of nucleic acids. Antimetabolites work by inhibiting important enzymes involved in nucleic acid synthesis or being incorporated into nucleic acids and generating the wrong genetic code. Both of these mechanisms cause inhibition of DNA synthesis until cell death. Based on their antagonistic properties, they are divided into folate antagonists (methotrexate, raltitrexed, pemetrexed), purine antagonists (6-mercaptopurine, 6-thioguanine, azathioprine), pyrimidine antagonists (cytosine arabinose, 5-fluorouracil, gemcitabine) (Muthalib, 2006).

2. GENOTOXIC AGENTS

As a genotoxic agent, this agent binds to DNA and or indirectly damages DNA by affecting enzymes involved in replication, which then induces apoptosis. This class of drugs is divided into:

2.1. Alkylating agents

This drug is an electrophilic compound in the body. The main mechanism is the interaction between electrophile molecules and DNA which causes substitution reactions, cross-linking, or DNA

strand breaks (Muthalib, 2006). Based on the structure and mechanism of covalent bonding, they are divided into several classes including nitrogen mustards (mechlorethamine, chlorambucil, cyclophosphamide, ifosfamide, and melphalan), nitrosoureas (streptozocin, carmustine, and lomustine), and platinum complexes (cisplatin, carboplatin, and oxaliplatin) (American Cancer Society, 2013; Page & Takimoto, 2015). The site of action of chemotherapy drug is shown in **figure 1**.

2.2. Intercalating agents *(anthracyclines)*

The mechanism of action is by causing topoisomerase II-dependent DNA cleavage and intercalation with double-stranded DNA. Drugs belonging to this group include doxorubicin, daunorubicin, epirubicin, and idarubicin (Muthalib, 2006; Page & Takimoto, 2015).

2.3. Topoisomerase inhibitors

These inhibitors are divided into topoisomerase I inhibitors and topoisomerase II inhibitors based on the enzymes inhibited. Topoisomerase I inhibitors (topotecan, irinotecan) inhibit topoisomerase I and disrupt the elongation phase of DNA replication. Topoisomerase II inhibitors (etoposide, teniposide, mitoxantrone) can form complexes with the enzyme DNA topoisomerase II, an enzyme that is important for completing DNA replication. This interaction causes the DNA strand to break and the cell cycle is inhibited to stop in the late S phase and early G2 phase (Muthalib, 2006; American Cancer Society, 2013).

3. MITOTIC INHIBITOR

Mitotic inhibitors include vincristine, vinblastine, vindesine, vinorelbine, and paclitaxel. Its activity is mainly due to its effect on microtubular proteins resulting in cessation of metaphase and inhibition of mitosis (anti-microtubules) (Muthalib, 2006).

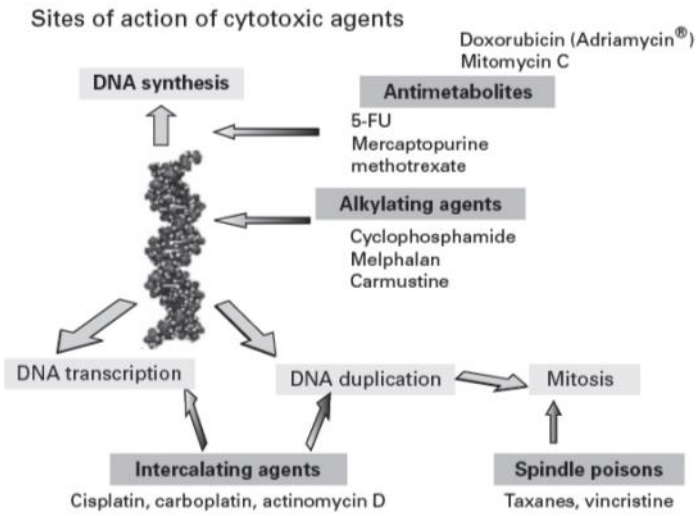

Figure 1. The mechanism and site of action of chemotherapy drugs (Luqmani, 2006).

Based on the mechanisms of the cell cycle, chemotherapy drugs can be grouped into: (Muthalib, 2006).

1. Drugs that are effective at a certain phase of the cell cycle (phase-specific drugs). Drugs that act in the S phase, for example, are antimetabolites that interfere with DNA synthesis, or topoisomerase I inhibitors that disrupt DNA structure. Drugs that act in the G2 phase are antibiotics (bleomycin), topoisomerase II inhibitors, and microtubule stabilizers or polymesators (paclitaxel). Drugs that act in

the M phase by interfering with chromosomal segregation are vinca alkaloids.

2. Drugs that are effective on cells that are in the cell cycle, but do not depend on the phase. Drugs that fall into this category are mostly alkylating agents and anthracyclines. These drugs are not non-specific because they still show greater effectiveness in one phase than in another, but not in the same level as phase-specific drugs.

3. Drugs that are effective both when cells are in the cell cycle or at rest (cell cycle–non-specific drugs). Drugs that fall into this group, for examples are nitrogen mustard and nitrosourea

In general, the treatment phases of acute lymphoblastic leukemia (ALL) can be grouped into induction, consolidation, and maintenance phases. The goal of induction therapy is to reduce the tumor burden by clearing as many leukemic cells as possible from the bone marrow. The induction therapy regimen based on BFM/COG (The Berlin-Frankfurt-Munster / a Children's Oncology Group) consists of a combination of 4 drugs including vincristine, anthracycline, corticosteroids, and L-asparaginase. The goal of consolidation (intensification) therapy is to remove potentially remaining leukemic cells after induction therapy. High-dose methotrexate, cytarabine, 6-mercaptopurine, and L-asparaginase are chemotherapy drugs used in consolidation therapy. The goal of maintenance therapy is to prevent recurrence after remission and consolidation therapy. Most maintenance regimens consist of a daily dose of 6-mercaptopurine and

a weekly dose of methotrexate (usually with periodic addition of vincristine and corticosteroids) for 2-3 years (NCCN, 2014).

Chemotherapy drugs can be divided into groups based on several factors such as how they work, their chemical structure, and their relationship to other drugs. Therefore, one drug can have more than one group. Based on the mechanism of action, chemotherapy drugs are divided into 3 categories: (American Cancer Society, 2013)

1. Stop the synthesis of a pre-DNA molecular skeleton.

This material works in some different ways. The building blocks of DNA consist of folic acid, heterocyclic bases, and nucleotides which are naturally formed in cells. All chemotherapeutic agents belonging to this group work by stopping several steps in the formation of nucleotides or deoxyribonucleotides (an important material for the formation of DNA). When this step is stopped, the nucleotides which are the building blocks of DNA and RNA cannot be synthesized, so cells cannot replicate because nucleotides are needed for the formation of DNA. The examples of drugs in this category are methotrexate, fluorouracil, hydroxyurea, and mercaptopurine.

2. Direct damage to DNA in the cell nucleus.

These substances damage DNA and RNA, by disrupting the DNA replication process and even completely stopping replication or causing the formation of dysfunctional DNA or RNA (new DNA or RNA does not code for anything). The examples of drugs in this class

include cisplatin and the antibiotics daunorubicin, doxorubicin, and etoposide.

3. Has the effect of synthesis or destruction of the mitotic spindle.

The mitotic spindle acts as a bridge between the north pole and south pole from when a cell starts to become 2 new cells. These spindles are very important because they separate the new DNA so that the offspring go to 2 new cells in each cell division. These drugs interfere with spindle formation and therefore disrupt cells. Examples of drugs of this class are vinblastine, vincristine, and paclitaxel.

CELL CYCLE

The cell cycle consists of 4 distinct phases: the G1 phase, the S phase (synthesis), the G2 phase, and the M phase (mitosis). The G1 phase, or gap phase, is where the cell grows and prepares for DNA synthesis. The S phase or the synthesis phase is the DNA synthesis phase of the cell. The G2 phase or the second gap, where the cell phase begins to divide, and the M phase of mitosis, where cell division occurs (Givan, 2001).

In the G1 phase, the cell receives the signals needed to synthesize the next RNA, the proteins needed to stimulate its growth. Metabolic changes prepare the cell for division. At some point, the cell divides and moves to the S phase when conditions are good and enters the S stage and begins DNA synthesis and replication. In the S phase, DNA synthesis occurs by doubling the genetic material. Semarang each chromosome contains 2 identical chromatids (Givan, 2001).

In the G2 phase, the cell continues its growth and prepares to carry out mitosis. The M phase itself consists of 2 processes: mitosis, in which the chromosomes in the cell nucleus are divided into 2 equal parts, and cytokinesis, in which the cytoplasm of the cell is divided into 2 parts in which each cell gets half. Nuclear division is followed by cell division. Cells that stop dividing for a while are called entering the quiescent stage or G0 phase. The period between mitosis and the next mitosis (i.e G1, S, and G2 phases) is known as interphase (Givan, 2001).

Regulation cyclin-dependent kinase (CDK)

The transition from one phase of the cell cycle to the next occurs in a routine, orderly manner and is regulated by a variety of different cellular proteins. The main controlling protein is the cyclin-dependent kinase (CDK), which is a family of serine/threonine protein kinases that are activated at certain points in the cell cycle. 9 CDKs have been identified to date, and 5 of them are active during the cell division cycle, for example, CDK4, CDK6, and CDK2 are active during the G1 phase, CDK1 in the G2 and M phases. When activated, CDK triggers the phosphorylation of certain proteins. CDK7 works in combination with cyclin H as CDK activating kinase (CAK). Other CDKs have not yet known their important role in normal cell cycle rate. CDK protein levels remain stable throughout the cell cycle, the opposite occurs in the activator protein, cyclin. Cyclin protein levels rise and fall during the cell cycle, this causes CDK activation to occur

periodically. Different phases of the cell cycle require different cyclins (Vermeulen *et al*., 2003).

There are 3 types of cyclin D (cyclin D1, D2, and D3), they bind to CDK4 and CDK6, the CDK-cyclin D complex is important for entry into the G1 phase. Unlike other cyclins, cyclin D is not expressed regularly but is synthesized as long as growth factor stimulation is present. Another cyclin in the G1 phase is cyclin E which binds to CDK2 to control the rate of the cell cycle from G1 to the S phase. Cyclin A binds to CDK2 and this complex is required during the S phase. In the late G2 phase and early M phase, cyclin A forms a complex with CDK1 to induce entry into the M phase. In the M phase, it is further regulated by cyclin B, which forms a complex with CDK1. A total of 16 cyclins have been identified, but not all cyclins play a role in the cell cycle (Vermeulen *et al*., 2003).

CDK activity can be inhibited by cell cycle inhibitory proteins, called CDK inhibitors (CK1) which bind to CDK alone or in the form of CDK-cyclin complexes, and function to regulate CDK activity. Types of CDK inhibitors include 2 different families, namely the INK4 family and the Cip/Kip family **(table 1)**. INK4 family inhibitors form stable complexes with CDK enzymes before binding to cyclins, preventing CDK binding to cyclin D. Cip/Kip family inhibitors inactivate CDK-cyclin complexes, specifically CDK1-cyclin B. p21 also inhibits DNA synthesis by binding to and inhibiting nuclear cell proliferation antigens / proliferating cell nuclear antigen (PCNA). Expression of p21 under the control of the p53 tumor-inhibiting gene.

The p21 gene promoter has a p53 binding site, causing p53 to activate the p21 gene. The expression and successive activation of p15 and p27 increase the TGF- response, causing growth inhibition (Vermeulen *et al.*, 2003).

Table 1. Cyclin-dependent kinase inhibitors (CKI) bind to CDK alone or the CDK-cyclin complex and regulate CDK activity. P19 (ARF) is also encoded by the INK4 locus, but its activity as a CKI is unknown (Vermeulen *et al*., 2003).

CKI family	Function	Family members	
INK4 family	Inaktivation of	p15	(INK4b)
	G_1 CDK	p16	(INK4a)
	(CDK4, CDK6)	p18	(INK4c)
		p19	(INK4d)
Cip/Kip family	Inaktivation of G_1 cyclin-CDK complexes and cyclin B-CDK1	p21	(Waf1, Cip1)
		p27	(Cip2)
		p57	(Kip2)

RESPONSE TO DNA DAMAGE

Under stressful conditions caused by exposure to chemotherapeutic agents, radiation, or environmental genotoxic agents such as polycyclic hydrocarbons or ultraviolet light, DNA damage is generally an early event. Double strand breaks (DSBs) or single-strand breaks (SSBs) are key defects that initiate the activation of the DNA damage response. Double-stranded DNA is more sensitive to exposure to

chemicals or nucleases than when it is separated into 2 single-stranded DNA. DSBs are produced directly or indirectly by a variety of anticancer drugs, including DNA intercalation, acylation or crosslinking agents, topoisomerase inhibitors, and nucleotide analogs. When DSBs occur, mutated ataxia-telangiectasia (ATM) is recruited by the MRE-11-Rad50-NBS1 (MRN) complex at the site of the damaged DNA and subsequently phosphorylates substrates such as checkpoint kinase 2 (Chk2) and p53. p53 causes transcriptional activation of many different functional programs, for example, cell cycle regulatory proteins such as p21 and proapoptotic factors such as CD95, PUMA, and BAX. Recent studies have also shown that p53 also causes non-transcriptional proapoptotic activation through regulatory pathways in mitochondria. In sublethal DNA damage, there are DNA repair mechanisms to maintain cell life. Sublethal DNA damage evokes a survival pathway through p21 which mediates cell cycle arrest. If the damage is too severe to repair, the system activates leading to cell death in response to cellular stress. Targets of activated p53 proapoptotic genes include Bax, Puma, Noxa, and Fas which trigger apoptosis. In SSB, ataxia-telangiectasia and Rad3 related (ATR) activation and Chk1 phosphorylation occur. Phosphorylated chk1 inhibits cdc25c, which causes the cell cycle to stop in the G2/M phase or cdc25a, which triggers the termination of the S phase (Fulda *et al*., 2010).

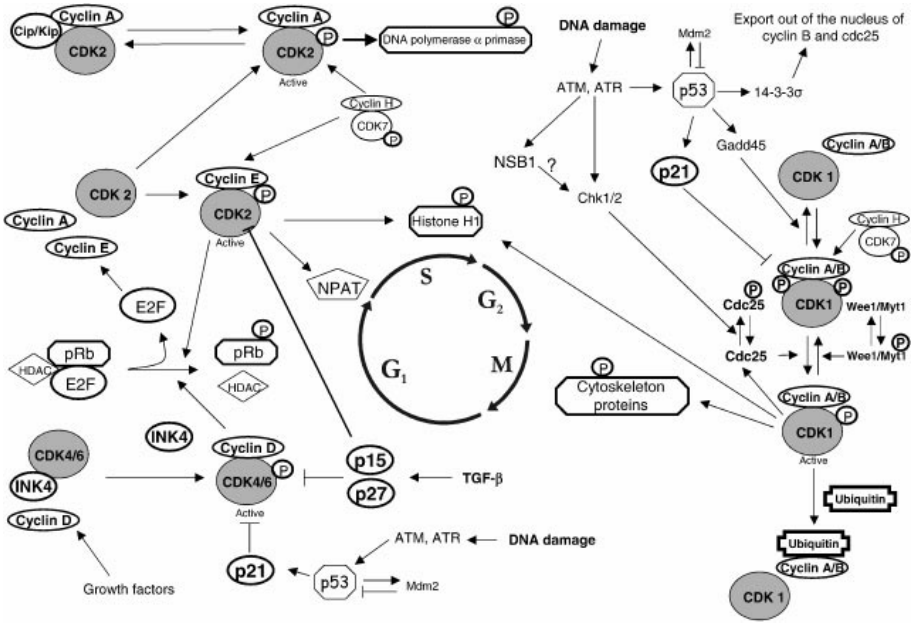

Figure 2. Schematic of several important steps in cell cycle regulation. P, phosphorylation side (activate, inhibit) (Fulda *et al*., 2010)

In mammalian cells, strict regulation is needed at each phase of the cell cycle so that the process of sending copies of the genome to identical derived cells can take place precisely. Cyclin-dependent kinases (Cdks) function to regulate cell cycle transitions and the activity of this Cdk is controlled by various positive and negative mechanisms. There are 2 main Cdk namely Cdk1 and Cdk2 which are expressed at constant levels during the cell cycle. For these cyclin-dependent kinases to be active they require linkage to the cyclin subunit and phosphorylation of threonine residues located in the T-loop domain. In contrast, phosphorylation of 2 specific amino acid residues (Tyr15 and Thr14) located within the ATP bond leads to the inactivation of Cdk1 and Cdk2. The protein kinase Wee1/Mik1/Myt1

also facilitates this inhibitory phosphorylation, while Cdc25 is responsible for dephosphorylation activation (Fulda *et al*., 2010).

Cdc25 was first identified in dividing fungi as a necessary factor for entry into mitosis. Thus, three mammalian genes that are complementary to the cessation of the G2 cycle were identified, i.e cdc25A, -B, and -C. These three genes encode phosphatases that can cause dephosphorylation of phosphotyrosine and phosphothreonine residues to activate their Cdk substrates. Cdc 25B and 25C regulate only the G2/M transition, while Cdc25A plays a role and is involved in more cell cycle transitions, namely early (G1/S) and late (G2/M) (Fulda *et al*., 2010).

The function of Cdc25A is to regulate the entry of the S phase after dephosphorylation of Cdk2. This was confirmed in a study that gave anti-Cdc25A microinjection causing the G1 phase to stop in cells, while this overexpression of Cdc25A led cells to enter the S phase and activate Cdk2. Cdc25A protein levels increase since the G1/S transition to mitosis **(figure 2)** (Fulda *et al*., 2010).

CHEMOTHERAPY RESISTANCE

Cellular drug resistance is known to be an important determinant of clinical outcomes after chemotherapy. The mechanism of resistance can occur because 1) drug transporter-mediated, 2) related to cell death and apoptosis mechanisms, 3) involving telomerase, 4) deficiency of DNA repair system, 5) contribution of leukemic stem cells (Su *et al.*, 2011).

Drug resistance associated with drug transporters is related to the role of membrane proteins in causing an efflux of cytotoxic substances, thereby reducing drug accumulation in cells and toxicity. Most of these transmembrane proteins belong to the ATP-binding cassette (ABC) family including P-glycoprotein (P-gp), the multidrug resistance-associated protein (MRP) family, breast cancer resistance protein (BRCP), lung resistance protein (LRP) (Su *et al*., 2011).

The P-gp expression occurs in approximately 30% of AML patients at diagnosis and >50% of relapses and is associated with reduced rates of complete remission and shortened patient life span. P-gp expression was also found in CML of blastic crisis, chronic lymphoblastic leukemia, multiple myeloma, non-Hodgkin's lymphoma, and ALL. In humans, P-gp is encoded by 2 MDR genes, namely MDR1 and MDR3 which are located on chromosome 7q21. P-gp is a 170 kDa polypeptide, consisting of 1280 amino acids. P-gp can be phosphorylated at several sites via several kinases, including protein kinase C and cAMP-dependent protein kinase A. Phosphorylation of P-gp is associated with drug resistance. Treatment with TPA phorbol ester that stimulated P-gp phosphorylation resulted in increased drug resistance and decreased drug accumulation in multiple drug-resistant cell lines. In contrast, protein kinase inhibitors, such as staurosporine, decrease phosphorylation and interfere with anticancer drug transport. P-gp has a variety of substrates. All of the substrates are large hydrophobic and amphipathic molecules. These molecules can slip into the membrane and enter the cytosol by passive diffusion. The substrate and P-gp interactions occupy space within the membrane. P-

gp mutational analysis indicated that some point mutations resulted in altered drug transport activity. This includes changes in Gly185Val causing decreased vinblastine transport but increased colchicine transport. Two different study groups reported that mutations in the main phosphorylation site in P-gp did not affect its transport function (Su *et al*., 2011).

Multidrug resistance-associated protein (MRP) encoded by the MRP1 gene located on chromosome 16p13.1, is a membrane-binding glycoprotein consisting of 1531 amino acids. In MRP1, the functions of NBD1 and NMD2 are not the same. NBD1 has a higher affinity for ATP than NBD2. When the substrate binds to the TMD of MRP, a conformational change of the MRP1 protein occurs, causing ATP binding to NBD1. It further changes the protein conformation and increases ATP in NBD2. When NBD1 and NBD2 are occupied by 2 ATP simultaneously, the bound substrate is transported out. MRP1 is expressed in almost all cell types and different organs. Unlike P-gp, which is located at the top of the epithelial cell membrane without exception, MRP1 is basolateral and tends to pump drugs into the body, rather than excrete it in the bile, urine, or intestines. Cells with overexpression of MRP1 protein are resistant to various anticancer drugs, such as doxorubicin, epirubicin, vinblastine, vincristine, and etoposide. The MRP1 protein works with glutathione (GSH) as a co-transport for hydrophobic anticancer drugs across biologic membranes (Su *et al*., 2011).

Lung resistance protein (LRP), also known as major vault protein (MVP), is often expressed at high levels in drug-resistant cell lines and tumor samples. Vaults are ribonucleoprotein (RNP) particles that are present in the cytoplasm of almost all eukaryotic cells and are thought to be involved in intracellular transport processes. Vault causes drug resistance by transporting out of the target drug intrasellar or trapping the drug. Several studies have shown that LRP/MVP is an independent prognostic factor for chemotherapy response. This protein is expressed in AML, multiple myeloma, and diffuse B-cell lymphoma and is associated with poor response to platinum chemotherapy or alkylating agents (Su *et al*., 2011).

Various data support the association of functional apoptotic pathways in cancer cells with chemotherapy sensitivity. The discovery of the bcl protein family is the latest proposed MDR mechanism. The apoptotic proteins bcl-XL and bcl-2 are strongly associated with drug resistance. Bcl-2 inhibits cell death and alters the ratio of normal cell death to cell division, allowing tumor cells to accumulate mutations, thereby making cells invasive and metastatic. Transfection with bcl-XL cDNA has been shown to protect several cell types in vitro against chemotherapeutic drug-induced apoptosis. Transfection of bcl-XL cDNA into a murine IL-3-dependent prolymphocyte cell line, FL5.12, was found to increase resistance to anticancer drugs bleomycin, cisplatin, etoposide, and vincristine (Su *et al*., 2011).

Telomerase is responsible for the rejuvenation of the ends of chromosomes called telomeres. Telomerase can inhibit the process of

cell aging and apoptosis by inhibiting telomere shortening. Telomerase activity, which is detected in most cancer cells, makes it maintain its proliferative capacity, resulting in the immortality of a cell which is the key to malignancy (Su *et al*., 2011).

Deficiency of DNA self-repair results in a high risk of malignant tumorigenesis. Defects in this system can lead to the accumulation of mutations in some proto-oncogenes or tumor suppressor genes, resulting in the transformation into cancer. It can be speculated that abnormalities in the DNA repair system increase the risk of multidrug resistance. Cell death and cell survival after DNA damage depend on the relative intensity of the generated signal and the communication between the associated effectors. . Among the effectors, the p53 tumor suppressor gene plays an important role in determining the ultimate fate of cells. DNA damage recognition proteins activate the mitogen-activated protein kinase signal transduction pathway, which then activates p53 function and causes the cell cycle to stop at the G2/M checkpoint for DNA repair. If the DNA damage is too extensive to repair, apoptosis occurs via the box and caspase systems (Su *et al*., 2011).

Cancer stem cells, like normal stem cells, can renew themselves and produce differentiated progenitors. These cancer stem cells have the container capacity to make secondary tumors, reflecting cancer initiation activity and therapeutic resistance. Stem cells are primarily characterized by an indefinite self-renewing portion, maintaining and multiplying a pool of undifferentiated cells throughout the life of the

host and differentiating into multiple lineages. Because, because stem cells and many cancer cells have the capacity for self-renewal and differentiation, it is proposed that tumors originate from mutated stem cells, called cancer stem cells. Cancer stem cells have been identified in leukemia. Many researchers now suspect that all cancers consist of a mixture of stem cells accounting for 1% of total tumor cells and proliferative cells, making these stem cells difficult to detect and study. Therefore, the presence of cancer stem cells is a source of recurrence and metastasis. ABCB1 and ABCG2 genes are expressed in almost all tumor stem cells (Su *et al*., 2011).

Multi-drug resistance can be caused by decreased drug entry into cells, abnormalities of intracellular metabolism of drugs into their active forms, increased drug inactivation, increased cellular repair mechanisms, changes in target molecules, changes in cell death regulators. Many drugs are used as leukemia chemotherapy regimens, and each drug has its own resistance mechanism, so there is no single mechanism that is responsible for clinical resistance (Su *et al*., 2011).

CELL PROLIFERATION EXAMINATION

Four procedures are widely used to analyze the cell cycle by flow cytometry. The first procedure detects 5'-bromo-2'-deoxyuridine (BrdU) that joins cells undergoing DNA replication. The second approach is based on bivariate analysis of DNA and protein content related to proliferation, namely cyclin. This approach can differentiate G0 cells from G1 cells, identify mitotic cells or other intracellular protein-associated expressions concerning cell cycle positions. The

third and fourth procedures were based on analysis of the DNA content of cells after staining cells with propidium iodide (PI) or 4',6'-diamidino-2-phenylindole (DAPI). This approach gives the distribution of cells in 3 main phases of the cell cycle (G1, S, G2/M) and makes it possible to detect apoptotic cells by their content of broken DNA (Pozarowski & Darzynkiewics, 2014).

1. The bromodeoxyuridine/propidium iodide method

Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) is a thymidine analog nucleoside, commonly used to detect cell proliferation in living tissue. This substance can enter into the newly synthesized DNA in cell replication during the S phase, replacing the position of thymidine during DNA replication. Specific antibodies to BrdU were then used to detect the fused BrdU, which further indicated that the cell was actively replicating. This antibody binding requires DNA denaturation (BrdU Flow Kits, 2014).

This method is a classic method. This procedure requires partially denatured DNA. This denaturation is necessary because BrdU antibodies will only bind BrdUs that are fused to single-stranded DNA. The remaining undenatured DNA was then stained with propidium iodide (PI). The green fluorescence generated from the fluorescent conjugated antibody essentially measures BrdU. The red fluorescence of the PI measures the DNA content. DNA denaturation can use heat or high molarity HCl. This method can be used for both fixed and non-fixed cells (Capri & Barbieri, 2014).

Because BrdU can replace thymidine during DNA replication, it can cause mutations, so its use has the potential to endanger health, however in vivo cancer cell proliferation studies using this material are still widely used.

2. Cyclins/propidium iodide method

Cyclins are a key component of driving the cell cycle rate. The expression especially cyclin D, E, A, and B1 plays an important role in initiating the cell cycle and cell division. In this procedure, cell cycle expressions were detected using specific monoclonal antibodies (mAbs) and analyzed for their DNA content. In general, cyclin D1 expression peaks can be detected at the beginning of G1, typical cyclin E peaks at the G1/S transition, cyclin A peaks can be detected during the G2/M phase, and typical cyclin B1 peaks at late G2/M. This method compared to other methods can distinguish the G0 phase from G1 and the G2 phase from M. It should be noted that not all cell types are the same (for example, cyclin D1 was detected not only in G0/G1 but also in G2/M, although very rare cells of this type).

Bivariate analysis between cyclin expression and DNA content makes it possible to distinguish between cells that have the same DNA content but are in different cycle phases, such as between cells in G2 and M based on differences in cyclin A content, or between diploid G2 and tetraploid cells G1 based on differences in the expression of cyclin E and/or B1. G0 cells did not express cyclin type D or E, while cells in the G1 phase had positive cyclin D and/or E expression.

3. Dying with propidium iodide (PI)

The principle of this test is to make the outer membrane of normal cells permeable by giving detergent or alcohol so that propidium iodide can enter the nucleus. The addition of RNase can be given if we want the double-stranded RNA not to contribute. A red filter and a photomultiplier tube are used to detect red fluorescence. Nuclear particle fluorescence results from normal, non-dividing cells will appear on the histogram as a single narrow peak, all particles emit an almost equal amount of red fluorescence, this supports the existing knowledge that the nucleus of all normal, non-dividing organisms contains a certain amount of red fluorescence. the same DNA (Givan, 2001).

The histogram of flow cytometry gives different results according to the presence of the nucleus. Based on the DNA content of the cell is divided into being in a cycle (not resting), in this situation we will find some nuclei with the amount of DNA 2N (cells in the G0 or G1 phase), some nuclei with the amount of DNA 4N (cells in the G2 or M phase), and some nuclei with different amounts of DNA spanning the 2N and 4N populations (Givan, 2001).

4. Dying with 4',6'-diamidino-2-phenylindole (DAPI)

The principle and staining of DAPI is the same as that of PI and the histogram formed is almost the same.

CONCLUSION

Chemotherapy drug resistance is basically determined from whether chemotherapy drugs can reach the target or whether cellular changes have occurred so the cells become resistant. There are various chemotherapy drugs with different mechanisms of action. The mechanism of action is differentiated based on the site of action and how the drug act, or what phase of the cell cycle which become the target of the drug.. Based on how the drug act in the cell cycle, chemotherapy drugs are distinguished: 1) Drugs that are effective at a certain phase of the cell cycle, 2) Drugs that are effective on cells along all the cell cycle, do not depend on the phase, 3) Drugs that are effective both the cell during in the cycle or at rest.

The cell cycle itself has 4 different phases, i.e the G1 phase, the S phase (synthesis), the G2 phase and the M phase (mitosis). The switch from one cycle to the next is controlled by protein cyclin dependent kinase (CDK) and CDK inhibitors (CKI). This cell cycle laboratory examination can be done by these methods: 1) bromodeoxyuridine/propidium iodide method, 2) Cyclins/propidium iodide method, 3) staining with propidium iodide (PI), or 4) 4',6'-diamidino-2-phenylindole (DAPI) with the advantages and disadvantages of each method.

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CHAPTER 9

THE EFFECTS OF PERCEIVED SOCIAL SUPPORT, PSYCHOLOGICAL RESILIENCE AND COPING STRATEGIES ON LIFE SATISFACTION IN MEN DIAGNOSED WITH TESTICULAR CANCER

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INTRODUCTION

Testicular cancer is the most common cancer seen in young men. This cancer, which is seen in early ages, affects the family life, career and especially sexual functions of men. With an adequate oncological care, it is most curable cancer with a more than 95% of survival rate (Atuhaire et al., 2019). In the studies conducted, psychological disturbances caused by sexual dysfunction and the thought of experiencing infertility problems in the future were found in patients diagnosed with testicular cancer (Carpentier et al., 2011). While high levels of anxiety and depression symptoms were detected in the patients, it was argued that these could have a negative effect on the quality of life of the patients (Xia et al., 2019). Testicular cancer and its diagnosis cause some psychological problems in patients. Patients who need psychological support both after the diagnosis and during the treatment process, not knowing the strategies to cope with the problems may cause some negative reactions (De Toni et al., 2019). The study result demonstrated that erection, ejaculation disorders, fear of infertility and body image problems occur frequently (DeRouen et al., 2016). Testicular cancer is a condition that creates problems with a major life crisis and psychological chronic stress in the diagnosis and medical treatment process among couples. Also, testicular cancer, which is a life crisis for individuals; it reduces the psychological resilience of individuals and partners, forces coping strategies, consumes their energy both physically and psychologically (Hanson et al., 2016). However, the diagnosis and treatment of testicular cancer also negatively affects the life satisfaction of individuals. For this

reason, the diagnosis and treatment process of cancer is a process in which individuals need more social support and want to perceive this support at a high level. Since the life expectancy of cured testicular patients is long, minimizing the effects on long-term health and quality of life are important goals (Friedman et al., 2014). Although it is predicted that patients' psychological resilience and life satisfaction and often psychological symptoms may be observed during the treatment process of testicular cancer, there is no study conducted on this subject.

This study aimed to investigate the effects of perceived social support, psychological resilience and coping strategies on the life satisfaction variable of patients diagnosed with testicular cancer. In addition, it was aimed to reveal the effects of depression, somatization and anxiety that patients have on life satisfaction.

METHODS AND MATERIALS

Study Design

The study was a descriptive and cross-sectional study using survey questionnaires to measure the outcome variables. A total of 174 men diagnosed with a testicular cancer at the age of 20-60 were included in the study. The survey method was used to collect data from the participants.

Instruments

Participants' socio-demographic characteristics such as age, marital status, educational level, income and other clinical characteristics were collected and evaluated.

Hospital Anxiety and Depression Scale (HADS)

The Hospital Anxiety and Depression Scale is a commonly use for the participants to measure anxiety and depression levels. Consisting of 14 items, HADS measures both anxiety (HADS-A) and depression (HADS-D) levels with an equal number of questions. Patients rank the question on a Likert scale ranging from 0 to 3 and the subscale from 0 to 21. A total score of 8 or above was defined as an optimal cut-off score for comfort for both anxiety and depression.

The Multidimensional Scale of Perceived Social Support (MSPSS)

Multidimensional Perceived Social Support Scale was developed to determine the social support elements perceived by individuals. It consists of 12 items in total. It is a 7-point (1-7 points), likert-type scale ranging from "absolutely no" to absolutely yes. The source of perceived social support can be measured in three sub-dimensions: family, friends, and significant others. The lowest score that can be obtained from the subscales is 4 and the highest score is 28. The lowest score to be obtained from the whole scale is 12, and the highest score is 84.

The Coping Attitudes Scale (CAS)

The scale consists of 60 questions and 15 subscales. Each of these scales provides information about a separate coping attitude. As a

result, the higher the scores to be obtained from the subscales give the possibility to comment on which coping attitude is used more by the person. Five of these coping attitudes (active coping, planning, suppressing other occupations, holding back, use of useful social support) are classified as problem-focused. The other five coping attitudes (use of emotional social support, positive reinterpretation and development, acceptance, joking, and religious coping) are classified as emotion-oriented. The other five coping attitudes (focusing on the problem and revealing emotions, behavioral disengagement, substance use, denial and mental disengagement) are classified as the least useful non-functional coping attitude.

The Satisfaction with Life Scale (SWL)

The Satisfaction with Life Scale was prepared by Diener et al. (1985) in order to determine the level of satisfaction with life and to conduct standard studies on this subject. This scale consists of 5 items that include the expressions: "My life is close to my ideals in many aspects, my living conditions are very good, I am satisfied with my life, I have achieved what I wanted so far, and if I were born again, I would hardly change anything in my life." It is a 5-point Likert type self-rating scale. Its scoring is between 5-25. It provides a measure of satisfaction for individuals in all their lives and all aspects of their lives. It gives people the opportunity to evaluate and rate themselves. If the individual gets a low score on the scale, it means that his / her life satisfaction is low and that he/she gets a high score means that his / her life satisfaction is high.

The Resilience Scale for Adults (RSA)

It is a 5-point Likert-type scale that includes a total of 33 items, in order to avoid biased evaluations in choosing the items, in which positive and negative features are on different sides. The dimensions in the scale are named as 'self-perception', 'future perception', 'structural style', 'social competence', 'family adaptation' and 'social resources'. In the assessment, the scoring method was allowed to measure psychological resilience high or low, and it was suggested that existing inverse questions should be evaluated according to this scheme. Friborg et al. (2003) were developed the scale.

Statistical Analyses

The obtain data was analyzed using the SPSS 23 package program. The descriptive analysis including mean, standard deviation, percentage and frequency were calculated. Logistic regression and multiple linear regression analysis were used to examine whether the study has a significant predictive effect on dependent variables. The statistical significance was taken as $p < 0.05$.

RESULTS

A total of 173 testicular cancer patients were included in this study with a mean age of 36 (SD 12.76). The majority of them (81.1%) were married, had tertiary education (41.9%) and low income (67.8%). The most of them were smoked cigarettes (62.6%) and used alcohol (72.9%). In addition, the majority of them had surgery (64.3%) and had no comorbidities (79.3%).

Table 1. Socio-demographic characteristics of participants

	n	%
Age (Ave+SD)	36 (12.76)	
Marital Status		
Single	33	18.9
Married	141	81.1
Education		
Primary	36	20.8
Secondary	65	37.3
Tertiary	73	41.9
Occupation		
Private	105	60.3
Government	57	32.7
Not working	12	7
Income		
Low	118	67.8
High	56	32.2
Smoker		
Yes	65	37.4
No	109	62.6
Alcohol		
Yes	47	27.1
No	127	72.9
Surgery		
Yes	112	64.3
No	62	35.7
Comorbidities		
Yes	36	20.7
No	138	79.3

In table 2, mean scores, standard deviation and ranges of domains of HADS, MSPSS, CAS, SWL and RSA were presented. The mean

HADS-anxiety score was 1.9 (SD=2.9) while the mean score of HADS-depression was 1.8 (SD=2.5). The CAS scores ranged from 8.0-63.0 with a mean of 22.9 (SD=8.7) indicating level of coping attitudes. The SWL scores ranged from 5.0-25.0 with a mean of 12.2 (SD=4.2) indicating mild level of life satisfaction among the participants. The highest mean score of family was 54.7 (SD=8.5) among MSPSS subscales, followed by 48.5 (SD=9.3) for friends and 53.3 (SD=5.8) for others. The RSA scores ranged from 22.0-62.0 with a highest mean 52.4 (SD=6.4) of social resources and lowest mean 47.5 (SD=8.2) of perception of self. Other scores and range of subscales of RSA were distributed between the highest and lowest mean scores along with the ranges.

Table 2. Mean, standard deviation and range of domains of HADS, MSPSS, CAS, SWL, RSA

Domains	Mean (SD)	Range
HADS		
HADS-Anxiety	1.9 (2.9)	0–14.0
HADS-Depression	1.8 (2.5)	0–13.0
MSPSS		
Family	54.7 (8.5)	29.8–64.2
Friends	48.5 (9.3)	25.1–56.3
Others	53.3 (5.8)	21.3–56.9
RSA		
Perception of Self	47.5 (8.2)	26.2–56.8
Planned Future	49.6 (10.2)	22.7–61.7
Social Competence	47.8 (10.8)	25.8–54.2
Family Cohesion	48.2 (8.1)	22.1–52.9
Social Resources	52.4 (6.4)	32.8–59.7
Structured Style	51.5 (6.2)	28.7–57.7
CAS total	22.9 (8.7)	8.0–63.0
SWL total	12.2 (4.2)	5.0–25.0

HADS-Anxiety: Hospital Anxiety Scale
HADS-Depression: Hospital Depression Scale
MSPSS: Multidimensional Scale of Perceived Social Support
RSA: Resilience Scale for Adults
CAS: Coping Attitudes Scale
SWL: Satisfaction with Life Scale

Table 3 presents multiple logistic regression to determine whether the independent variables had an effect on the result. Analyzes were performed using the enter model in multiple linear regression analysis. The significance and explanation percentage of the model with all variables were calculated. In the regression analysis, the model was created with the stepwise variable selection method. The modeling process was performed with the independent variables of HADS anxiety, HADS depression, MSPSS, CAS, SWL and RSA. These independent variables were scored with the calculations specified in the scales. Accordingly, the model was found to be significant as a result of the variance analysis of the regression equation obtained as a result of the analysis ($F = 6.138$; $p < 0.05$). Accordingly, the variables of HADS depression, HADS anxiety, perceived social support, psychological resilience and coping strategies included in the multiple linear regression analysis explained the change in life satisfaction variance by 42% ($R^2 = 6.351$; $R^2 = 0.423$) determined ($p < 0.005$). The statistical significance was analyzed of the independent variables and the result showed that HADS depression score ($t = -0.31$; $p=0.000$), HADS anxiety ($t = -1.07$, $p = 0.002$) and psychological resilience ($t = -0.23$, $p = 0.001$) were found to be significant in terms of life satisfaction levels of patients treated with testicular cancer. There was no statistical significance found among other variables and life satisfaction levels of patients ($p>0.005$).

Table 3. Multiple logistic regression analysis of HADS, MSPSS, CAS, SWL, RSA

Domains	B	Std. error	**t**	p value	95% CI for B
HADS-Anxiety	–1.07	0.46	–3.37	0.002	–1.70 to –0.44
HADS-Depression	–0.31	0.61	–0.73	0,000	–1.14 to –0.53
MSPSS	–0.51	1.64	0.38	0,455	–0.42 to –0.03
CAS	0.04	0.06	0.64	0,182	–0.08 to –0.16
SWL	0.17	0.18	0.88	0,922	–0.14 to –0.36
RSA	–0.23	0.1	0.19	0,001	–0.42 to –0.03

R^2: 6.351; Adjusted R^2: 0.423; F = 6.138;
$p<0.05$

DISCUSSION

Testicular cancer is the most common type of cancer in almost 1% of men between the ages of 20 and 40 (Li et al., 2020). The most common problems faced by patients diagnosed with testicular cancer are sexual function problems. However, the most common mental problems among patients were stated as anxiety and depression. Factors such as the diagnosis of cancer, age, family history, smoking, alcohol use and psychological endurance were among the important factors affecting the treatment process of the disease (Parekh et al., 2020). The ages of the participants in the study ranged from 20 to 60 and the mean age was 36. The education level of patients diagnosed with testicular cancer is very important in terms of treatment process, reproductive and health behaviors. This variable affects the perception

of cancer and the level of cancer-related problems (Peters et al., 2008). For this reason, those with a high level of education may be more conscious during the treatment process. When the educational status of the participants was evaluated, it was found that most of the participants were tertiary (41.9%). The low education level of individuals; it is thought to lead to a decrease in their quality of life, less awareness of health risks, not knowing about health protective measures, not being able to use coping strategies when they experience health problems, and a decrease in their psychological resilience. The results from this study show that the majority of men diagnosed with testicular cancer patients report high level of anxiety and depression symptoms. Also, the results of the study indicate an increased occurrence of psychological resilience after a diagnosis of testicular cancer (Roy and Casson, 2017). There was a significant different between the patients who had diagnosed with testicular cancer, levels of depression, anxiety and psychological resilience. Although, there is a gap in the literature regarding this subject and testicular cancer patients, previous studies reported that psychological problems such as high level of anxiety and depression symptoms are related to testicular cancer patients during diagnosis and treatment process (Rusner et al., 2014). The study results have shown that psychosocial factors such as emotional disorders, spouse problems, lack of social support or social exclusion accompanying patients' increased stress can lead to a decrease in life satisfaction (Rovito et al., 2021). In addition, other studies reported that the social support perceived by cancer patients from their spouses has positive effects on

life satisfaction. Social support directly affects life satisfaction in cancer patients' coping with the disease during the treatment process (Xia et al., 2019). It has been reported that the level of psychological resilience due to stress and anxiety decreases in patients diagnosed with testicular cancer and treated (Tuinman et al., 2010). Thus, this has a negative effect on the quality of life. The psychological resilience of the patients is related to different variables in the treatment process and the importance of supporting psychological resilience in coping with the negativities experienced by the individuals and increasing their life satisfaction. In this study, it was found that resilience has a positive effect on life satisfaction. Although there are no studies on the low relationship between testicular cancer, which is one of the traumatic and negative life events, and life satisfaction, it has been reported in other cancer studies that individuals with more coping strategies have more positive life satisfaction. It has been reported that coping strategies directly contribute to changes in life satisfaction and are likely to change the level of life satisfaction.

CONCLUSION

Testicular cancer, which is common among men, causes negative consequences on the life of individuals. Social support of the patients both during the diagnosis period and during the treatment, it causes changes in life satisfaction and psychological resilience. Therefore, psychological problems such as a decrease in the coping strategies of the patients, depression or anxiety are beginning to be observed.

Testicular cancer is not only an oncological disorder, but also psychologically, and patients should be given support to cope with psychological disturbances and increase their psychological resilience.

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CHAPTER 10

DOSIMETRIC COMPARISON OF BEAM ARRANGEMENT IN INTENSITY-MODULATED RADIOTHERAPY

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INTRODUCTION

In adults among the primary brain tumors, the most common tumor is metastatic tumors, followed by glial tumors (Ostrom, 2013). Malignant gliomas (WHO Grade 3-4) constitute more than half of primary brain tumors (Rosell 2008, Ammirati 1987). The primary standard treatment for glioblastoma multiform (GBM) treatment is surgery (Fine, 1994). Radiotherapy has an important role in the treatment of GBM (Mann, 2017). The aim of radiotherapy is to deliver a radiation dose high enough to control the target while minimizing the radiation dose to the surrounding critical structures. In intensity-modulated radiotherapy (IMRT) aims to deliver uniform doses to the planning target volume (PTV), while sparing damage to normal tissues and organs at risk (OARs). The IMRT technique is among the commonly used radiotherapy techniques. (Wijsman, 2017). Radiotherapy treatments are becoming an increasingly complex process. Manual optimization (MO) for treatment plans takes more time depending on the quality of the plan (Tol 2012, Krayenbuehl 2015). The increasing complexity of treatment plans complicates the optimization procedure and thereby augments the rate of inconsistency between manually derived treatment plans (Hazell, 2016). Several trial-and-error optimization processes are usually required to achieve clinically acceptable plans. More manual actions could influence consistency and plan quality of the manual treatment plans. In order to improve the overall plan quality and to decrease the time required for planning, semi-automated planning algorithms have been developed.

(Fogliata, 2014). The optimal choice of beam irradiation directions beam angle optimization (BAO) can play an important role in IMRT treatment planning by improving organ sparing and tumor coverage, increasing the treatment plan quality. BAO is the use of a specific optimization algorithm to select the optimum angles of static beams, either coplanar or noncoplanar. The beam arrangement for intensity modulated radiotherapy (IMRT) have significant influence on treatment plan quality. This study aimed to compare the advantages of IMRT plans generated by the manual beam optimization (MO) and beam angle BAO techniques of treatment planning system (TPS).

MATERIAL AND METHOD

In this dosimetric study, 10 patients previously treated for glioblastoma multiform (GBM) was selected. All patients were immobilized with a head and neck thermoplastic mask. Computed tomograpy (CT) scan with a 3 mm slice thickness were transferred to TPS. Clinical target volume (CTV_{50}) was created by adding an isometric 2-2.5 cm margin to the gross target volume (GTV_{50}) to obtain the CTV, and the PTV_{50} was created by adding a 0.5 cm margin around the CTV_{50} for the PTV definition. For boost area, GTV_{60} was contoured using preoperative MRI axial T1 contrast images. PTV_{60} was created by adding an isometric 2-2.5 cm margin to GTV_{60} and 0.5 cm margin around CTV_{60} (Niyazi 2016, Kruser 2019). Optic chiasm, brain stem, optic nerves, right and left eyes, right and left lenses were contoured as organ at risk (OARs). All plans, the PTV_{50} received 50 Gy over 25 fractions with 2 Gy as a dose per

fraction, while the PTV_{60} planned 60 Gy over 25 fractions with 2.4 Gy as a dose per fraction. Manual optimization (MO) plans were prepared using five fields in coplanar arrangement. In the second plan, the selection of optimal gantry angle was selected by the algorithm used in the BAO. BAO was used with plan geometry optimization (PGO) algorithm (Litoborska, 2012) compatible with eclipse TPS. The total number of beams used in BAO plans ranged between 5 and 7 depending on the beam selection process by the optimization algorithm. IMRT plans were created in TPS 15.1 with a 6 MV photon beam and sliding window technique. All plans were compared based on the dose-volume histograms (DVHs). The 100% of the PTV were covered by 95% of the prescribed dose. Maximum and mean dose (D_{max} and D_{mean}), the average dose delivered to 98% (D98%), 50% (D50%), 2% (D2%), HI, CI and MU were compared for the for the primary tumor. For the brain stem, optic chiasm, optic nerves and eyes, D_{max} and D_{mean} doses were compared. The HI formula was defined according to the ICRU Report No:83. HI defined as:

$$HI=\frac{(D_{2\%}-D_{98\%})}{D_{50\%}}$$

used to quantify dose homogeneity in the PTV (ICRU Report 83, 2010). As the HI value decreases, the homogeneity of the targeted volume increases. In cases where CI is equal to 1, we can talk about the ideal dose distribution. If the CI is greater than 1, the irradiated volume is larger than the target volume, and if it is less than 1, the target volume is partially irradiated. The CI index is used to estimate

the degree of suitability of the plan (ICRU Report 50, 1993). It is calculated as the ratio of the volume of PTV, which receives 98% of the dose, to the total volume of PTV. This value was calculated automatically with the planning option. Paired sample test was used for descriptive statistics as well as determining normality of the distribution of the data. A p value of < 0.05 was considered to be significant.

RESULTS

The comparison for PTV, HI, CI and MU were tabulated in Table 1. According the results from this study D_{mean} doses for PTV_{60} were 60.58±0.32 and 60.24±0.37 for MO and BAO. The planned PTV_{60} in both groups reached 95% of the volume. There was not a significant difference to D_{max} (Gy) and D_{mean} (Gy) doses of the PTV_{60}. Figure1. are showed the evaluation of the MO plan with BAO plan. The conformity index (CI) values were 0.35±0.06 for MO and 0.36±0.07 for BAO. Homogeneity index (HI) values for MO was 0.13±0.01 and for BAO was 0.14±0.03, respectively. The monitor units (MUs) for MO and BAO were 860±111 and 752±136, respectively. BAO plans had a lower MUs values than the EBO plans ($p= 0.027$). In Table 2 were listed the summary of the dosimetric results of the OARs. DVHs comparison were showed for PTV_{60} and OARs in Figure 2. When the MO plans were compared with BAO plans, the D_{max} doses received by the OARs were similar. However, D_{mean} doses for OARs were significantly lower for BAO plans than MO ($p<0.05$).

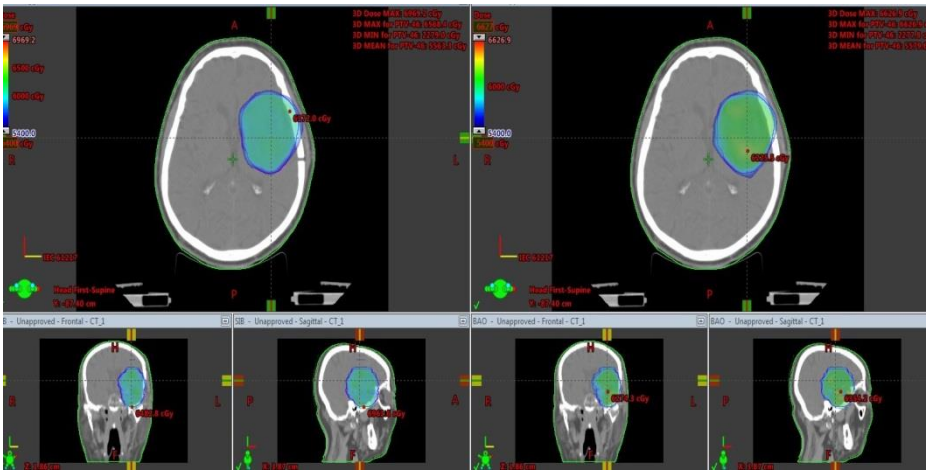

Figure 1. Isodose Curves of One Representative Patient for MO And BAO

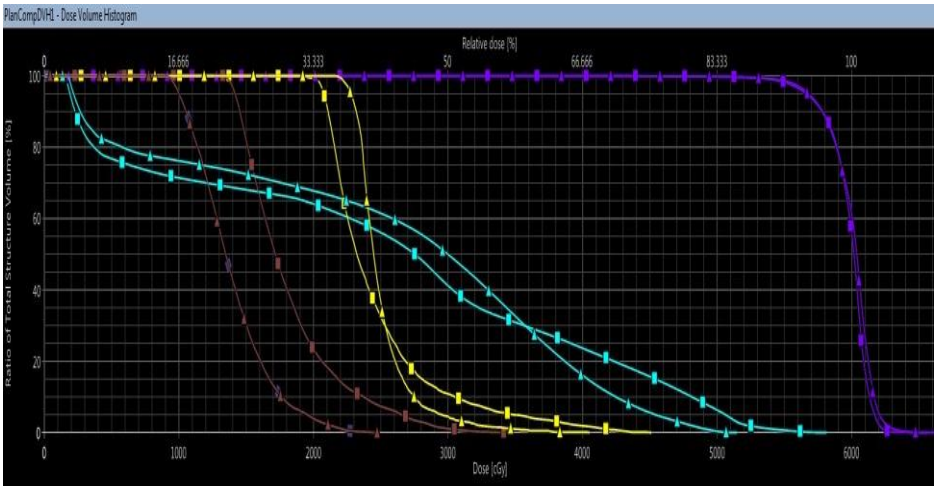

Figure 2. Dose-Volume Histogram Comparison of a Patient Purple: Planning Target Volume, Yellow: Optic Chiasm, Brown: Left Optic Nerve, Blue: Brainstem ■: Manual Optimization ▲: Beam Angle Optimization

Table 1. Dose Statistic Comparison for Planning Target Volume

Parameters	MO (Mean±SD)	BAO (Mean±SD)	p
PTV_{60} D_{98} (Gy)	57.80±0.20	57.84±0.32	0.183
PTV_{60} D_{95} (Gy)	58.42±0.15	58.21±0.17	0.245
PTV_{60} D_{50} (Gy)	60.42±0.15	60.23±0.21	0.354
PTV_{60} D_{2} (Gy)	62.32±0.21	62.45±0.11	0.804
PTV_{60} D_{max} (Gy)	63.60±0.44	63.87±0.52	0.751
PTV_{60} D_{mean} (Gy)	60.58±0.32	60.24±0.37	0.526
CI	0.35±0.06	0.36±0.07	0.251
HI	0.13±0.01	0.14±0.03	0.374
MU	860±111	752±136	**0.027**

Table 2. Dose Statistics Comparison for Organs At Risk

Parameters	MO (Mean±SD)	BAO (Mean±SD)	p
Brainstem D_{max} (Gy)	51.43±1.04	51.55±1.12	0.848
Brainstem D_{mean} (Gy)	19.18±5.86	17.15±4.87	**0.011**
Optic chiasm D_{max} (Gy)	42.41±7.23	42.56±7.23	0.285
Optic chiasm D_{mean} (Gy)	26.11±7.13	24.41±6.17	**0.020**
Left optic nerve D_{max} (Gy)	42.59±8.62	43.11±4.22	0.374
Left optic nerve D_{mean} (Gy)	28.45±5.43	26.71±3.78	**0.013**
Right optic nerve D_{max} (Gy)	15.73±4.77	15.88±3.54	0.507
Right optic nerve D_{mean} (Gy)	10.83±3.32	8.57±2.28	**0.011**
Left eye D_{max} (Gy)	36.36±9.11	37.14±7.23	0.321
Left eye D_{mean} (Gy)	21.16±8.13	20.84±7.42	0.274
Right eye D_{max} (Gy)	14.37±3.66	13.42±3.21	0.345
Right eye D_{mean} (Gy)	5.32±2.56	4.14±1.96	0.378

DISCUSSION

In the cancer treatment, the aim of radiotherapy treatment plans are to deliver a high enough dose of radiation to the primary tumor while minimizing less damage to critical organs. In this study, we compared MO and BAO plans dosimetrically to find the optimal plan for glioblastoma multiform radiotherapy. According to the results of our study, we found similar target coverage, HI and CI in MO and BAO plans. Hovewer, the BAO plans had lower mean OARs dose with compared to MO plans. Our study shows that the IMRT plans based on optimal selection of beam angles has sufficient PTV and superior OARs sparing to the manual beam selection. When compared MUs between the two techniques, the BAO plans significantly gave better results than MO plans because of the MUs are significantly lower in BAO. Lower MUs are seen as a potential advantage for breath control during radiotherapy. Reducing MUs will shorten the overall treatment time for patients.

Yousif et al. compared the BAO for 3 DCRT treatment planning (Yousif, 2021). They stated that the DVH differences for the two techniques did not have any clinical significance. PTV D_{max} for both techniques was comparable but the average MUs plans were found to be lower for BAO. They suggested that BAO provides good quality conformal plans. Also they indicated that BAO plans reduce planning time. The study we conducted in parallel with the study showed that BAO plans reduce the treatment time. However, BAO plans have been found to be more advantageous in sparing critical organs.

Shukla et al.compared the dosimetric advantages between equiangular beam optimization (EBO) and BAO plans (Shukla, 2016). They found that when BAO compared to EBO, OARs receive almost identical or slightly better doses in BAO plans. They stated that for two plans, CI and HI values were almost similar. However, they have noticed that, for the BAO plans were significant reduction in MUs. They suggested that BAO enables superior plan with respect to MUs and should be used whenever possible in IMRT planning. Our study found a parallel result with this study.

Srivastava et al. compared the dosimetric benefits of MO and BAO (Srivastava, 2011). They selected prostate and head and neck patients for IMRT plans. They found that the DVH for target are almost identical for both techniques. However, for the OARs, BAO plans showed superior sparing compared with MO plans. Also, BAO plans produced statistically significant lower MUs. As a result of the study, the suggested that BAO plans provide advantage over MO. Our study showed similar resulted with this study.

Leung et al. compared the dosimetric advantages of beam arrangement methods by employing EBO, BAO, and volumetric modulated arc therapy (VMAT) in the head and neck (H&N) radiotherapy treated with IMRT (Leung, 2019). They found that there was no significant difference in conformity index for larynx cancer. They inicated that BAO techniques found higher CI for cancers of maxillary sinus. Contrary to this study, our study and other investigated studies have found similar CI values for both techniques.

CONCLUSION

In this study the advantages of BAO for GBM patients were addressed. This dosimetric study reveals the advantages of the BAO technique compared to the MO technique in IMRT plans. BAO plans provide nearly identical or better sparing to OARs. Also, BAO plans enable to reduce in MUs. The reduction in MUs directly affects the duration and efficiency of the treatment.

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CHAPTER 11

EVALUATION OF CONVENTIONAL MULTILEAF COLLIMATOR BASED STATIC AND DYNAMIC IMRT

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INTRODUCTION

In the worldwide, cervical cancer remains the most common gynecologic cancer and the fourth most common malignancy in women. (1) Chemical, hormonal, or other carcinogens may be implicated in cervical cancer. The aim of radiotherapy is to enable the best dose conformation to the target volume, while sparing healthy tissues and critical organs. (2,3). Developments in technology ensured true three dimensional (3D) tailoring of radiation fluence with clinical equipment and a planning time scale feasible for clinical application. Since its introduction into clinical use, IMRT has created widespread utilization (4,5). IMRT provides the dose modulation using two MLC-based dose delivery methods (static or step-and-shoot MLC (SMLC) and dynamic MLC (DMLC). The S-IMRT technique switch off the beam while the MLC leafs are moving. This method of IMRT delivery is also called "step and-shoot" (6,7). The accelerator starts to shoot the beam only when the MLC leafs attain their calculated positions. The SMLC technique forms discrete fluence maps with various intensity levels (ILs) to showed a targeted continuous fluence. However, the D-IMRT technique modifies the beam intensity by moving each MLC leaf with individual speed while the beam delivery continues. The method is known as D-IMRT and is called "sliding window" (8,9).

This study aims to to compare dosimetric variations of using the two IMRT delivery methods, S-IMRT and D-IMRT. IMRT plans were generated with 6 MV using D-IMRT and S-IMRT with 5 (IL5), 10 (IL10) and 15 (IL15). For each patient treatment plans were compared

with respect to the doses received by the organ at risk (OAR) including rectum, bladder, entire bowel, right femur and left femur and total monitor unit (MUs).

MATERIAL AND METHOD

Ten patients with historically confirmed cervical cancer were selected in this study. The patients were immobilized in a supine position and computerized tomography (CT) images were obtained with a 3 mm slice thickness. The images obtained from CT was transferred to planning system. Planning target volume (PTV) was delinated according to recommendations of consensus guidelines using Varian Eclipse 15.1 treatment planning software (10). Normal tissues including the rectum, bladder, entire bowel and bilateral femurs were contoured OARs. IMRT plans were generated using Varian DHX linear accelerator, which is capable of delivering both Static and Dynamic IMRT with 80-leaf MLC system. For the D-IMRT and S-IMRT technique, we planned seven treatment fields and 6-MV photons designed to treat. The prescribed dose to the target volume was 5040 cGy in 28 fractions. The optimization aim was to provide at least 95% of the PTV to receive prescribed dose. Firstly, IMRT plans were created using the D-IMRT method. Then, all plans were recalculated using the S-IMRT method to compare both techniques. S-IMRT plans were created for three (ILs) of 5, 10, and 15. All dose volume histograms (DVHs) obtained from D-IMRT and S-IMRT plans were evaluated for target volumes and critical structure. According to the The International Commission on Radiation Units

and Measurements (ICRU) 83 report, the evaluation of plans were performed based on the dose volume histogram derived from plans dose distributions (11). Maximum and mean dose (D_{max} and D_{mean}), HI, CI and MU were compared for PTV. The values of interest in this study included D_{max}, $D_{mean,}$ $V_{50Gy,}$ $V_{45Gy\ and}$ V_{30Gy} for the rectum, D_{max}, $D_{mean,}$ V_{50}, V_{45Gy} and V_{30} for bladder, $D_{mean,}$ V_{30} for right and left femur, D_{mean} for bowel were evaluated. Paired sample T-test was used and $p<0.05$ was considered to indicate a statistically significant difference.

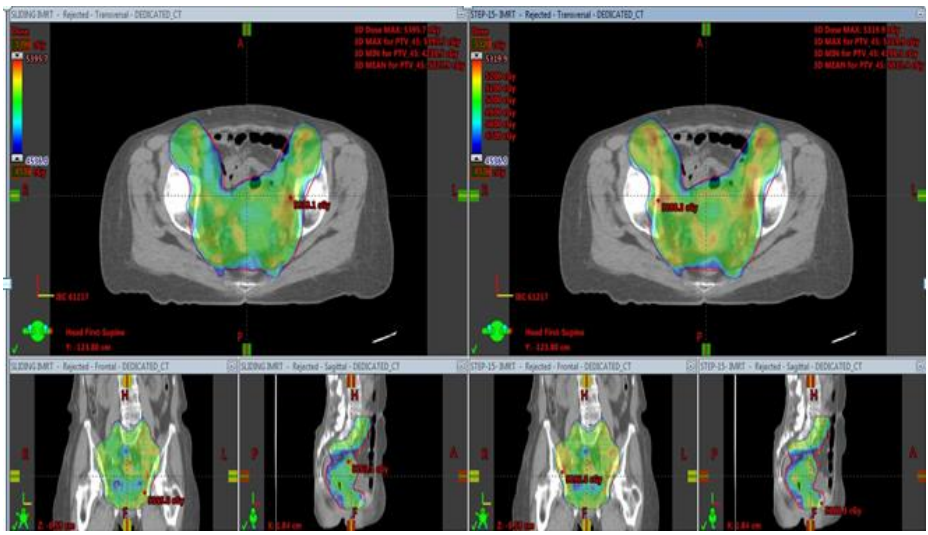

Fig 1. The Evaluation Isodose Curves of One Representative Patient for D-IMRT With S-IMRT with 15 (IL) (Eclipse treatment planning system in the Selcuk University Radiation Oncology)

RESULTS

The dosimetric comparison for PTV with standard deviation are listed in table 1. OAR according to four plans are tabulated in Table 2. For this study, the results of the D-IMRT plans were similar that of the S-IMRT plans with 5,10 and 15 (IL). The average mean PTV doses were 50.31±0.23, 50.18±0.45, 50.02±0.40 and 50.01±0.29 for D-IMRT, S-IMRT plans with 5, 10 and 15 (IL). There were a statistically significant differences found with 5 (IL) static plan with max dose measurements. The maximum PTV for the S-IMRT 5 (IL) was 55.47±0.38 when the D-IMRT, S-IMRT plans with 10 and 15 (IL) plans were 53.79±0.68, 53.79±0.98 and 53.18±0.48, respectively (p=0.002). PTV had similar result in HI and CI. The mean MU counts required for D-IMRT, S-IMRT plans with 5, 10 and 15 (IL) were 998±82, 1022±71, 04±41 and 1012±68, respectively. Figure 1. are showed the evaluation of the D-IMRT plan with S-IMRT 5 (IL) plan. Figure 2 are showed the comparison of the D-IMRT plan with S-IMRT 15 (IL) plan. As the number of ILs increased, the MU are decreased gradually in S-IMRT plans. The dynamic IMRT plan delivers the more MU compared to the static IMRT plan of 5, 10 and 15 (IL). According to the results of the study, OARs were almost similar results for the D-IMRT and S-IMRT plans with 5, 10 and 15 (IL). In D-IMRT plans, for the rectum and bladder V50Gy, V45Gy and V30Gy doses plans were slightly higher than S-IMRT plans with 5, 10 and 15 (IL). The S-IMRT plans with 5 IL delivery slightly degraded the PTV dose uniformity while increasing the high dose

volume in the rectum and bladder. Figure 3. are showed DVH comparison of the D-IMRT with S-IMRT with 5, 10 and 15 (IL).

Table 1. Dose Statistic Comparison For Planning Target Volume for A Total of 20 Prostate Patients

Parameters	D-IMRT	S-IMRT 5 levels	S-IMRT 10 levels	S-IMRT 15 levels	P (DS5L)	P (D-S10L)	P (D-S15L)
PTV D_{max} (Gy)	53.79±0.68	55.47±0.38	53.79±0.98	53.18±0.48	0.002	0.994	0.871
PTV D_{mean} (Gy)	50.31±0.23	50.18±0.45	50.02±0.40	50.01±0.29	0.579	0.228	0.232
D_{98}% (Gy)	48.39±0.30	47.60±0.47	48.52±0.92	48.18±0.43	0.009	0.314	0.332
D_{95}% (Gy)	48.97±0.23	48.08±0.39	48.28±0.79	48.65±0.38	0.003	0.041	0.121
D_{50}% (Gy)	50.36±0.35	50.49±0.35	50.20±0.53	49.84±0.17	0.619	0.407	0.002
D_2% (Gy)	51.90±0.40	53.25±0.62	52.41±0.91	51.36±0.37	0.021	0.360	0.298
HI	0.066±0.008	0.10±0.029	0.093±0.032	0.060±0.006	0.070	0.121	0.235
CI	0.44±0.17	0.52±0.09	0.41±0.21	0.22±0.14	0.442	0.713	0.009
MU	998±82	1010±71	1012±41	1024±68	0.422	0.808	0.512

Table 2. Dose Statistic Comparison for Organs At Risk for A Total of 20 Prostate Patients

Parameters	D-IMRT	S-IMRT 5 levels	S-IMRT 10 levels	S-IMRT 15 levels	p (D-S5L)	p (D-S10L)	p (D-S15L)
Rectum Dmax (Gy)	51.32±0.69	52.05±0.49	50.91±1.10	50.43±0.69	0.101	0.540	0.016
Rectum Dmean (Gy)	41.47±1.67	41.39±1.93	41.18±1.76	41.07±1.86	0.749	0.370	0.038
Rectum V30 (Gy)	90.13±5.62	89.96±6.05	89.86±6.02	89.93±6.13	0.541	0.283	0.478
Rectum V45 (Gy)	56.71±8.31	51.06±11.11	51.33±9.42	51.17±9.08	0.232	0.151	0.000
Rectum V50 (Gy)	2.68±1.78	1.67±2.75	1.32±2.49	0.11±0.23	0.076	0.981	0.122
Bladder Dmax (Gy)	52.04±0.67	52.89±0.86	51.68±0.93	51.24±0.74	0.044	0.506	0.504
Bladder Dmean (Gy)	44.55±1.16	44.38±1.01	44.22±1.19	44.10±1.13	0.455	0.301	0.293
Bladder V30 (Gy)	96.96±3.74	96.86±3.60	97.04±3.59	96.86±3.74	0.731	0.498	0.970
Bladder V45 (Gy)	57.65±11.59	54.39±13.42	54.35±12.17	52.05±14.61	0.102	0.104	0.025
Bladder 50 (Gy)	3.23±3.63	2.94±4.34	2.72±2.89	1.05±1.83	0.095	0.799	0.035

Right femur mean (Gy)	25.39±1.84	25.35±1.87	25.38±2.00	25.29±2.01	0.779	0.994	0.652
Right femur V30 (Gy)	32.31±4.01	29.15±5.02	29.32±5.08	29.36±5.19	0.395	0.419	0.431
Left femur mean (Gy)	26.45±1.62	26.35±1.60	26.27±1.65	26.02±1.71	0.696	0.308	0.649
Left femur V30 (Gy)	33.07±5.19	32.83±5.61	32.20±5.17	32.38±5.65	0.081	0.097	0.416
Bowel Dmean (Gy)	21.50±6.64	21.27±6.51	21.26±6.70	21.25±6.75	0.110	0.080	0.071

DISCUSSION

The IMRT plans requires important attention of planning, QA and dose delivery. In this study, we compared D-IMRT and S-IMRT plans with 5, 10 and 20 (ILs) in terms of PTV, MU and OAR. The D-IMRT fluence is delivered as it is created by the TPS, in the S-IMRT plans delivery, the fluence created by TPS is transformed to discrete ILs before the treatment, so the transformation causes S IMRT to lack in the targeted dose distribution. PTV max dose measurements were significantly affected in S-IMRT plans with 5 (IL) plans compared to D-IMRT and S-IMRT with 10 and 15 (ILs) plans and the obtained results were comparable to D-IMRT technique. When the the (IL) increased in S-IMRT plans; the intended fluence is delivered with less deficiency compared to 5 level S-IMRT plans, so when all the data obtained are examined, there is no much difference was observed D-IMRT plans and S-IMRT plans with 10 and 15 (ILs). From the obtained results, S-IMRT plans with 10 and 15 (ILs) give comparable results with D-IMRT plan and S-IMRT with 5 (IL) significantly affects target coverage. When the OAR doses were examined, there were no significantly differences observed between D-IMRT and S-IMRTplans with 10 and 15 (ILs). In all plans D-IMRT had lower doses when compared to S-IMRT plans with 5, 10 and 15 (ILs).

Manikandan et al. compared the effects of the number of (IL) on treatment outcome of S-IMRT plans with D-IMRT plans (12). They found that there were no significant differences between SMLC with 10 and 20 (IL) and D-IMRT plans but there were significant differences found with 5 (IL) S-IMRT compared to D-IMRT. They indicated that there were no significant differences found to be in normal tissue dose between S-IMRT and D-IMRT techniques and the MUs were more for D-IMRT compared with S-IMRT for all (ILs). Our results were similar with this research.

Chui et al. compared the effects of D-IMRT plans and S-IMRT plans with different number of (ILs) (13). They demosrated that 10 (IL) S-IMRT plan produced results similar to that from a D-IMRT plan. In S-IMRT plan, PTV coverage was improved by increasing the number of (ILs) and OARs were better protected. They found that the D-IMRT plan delivers the more MU compared to the S-IMRT plan of 5, 10 and 20 (ILs). Our results from the study were similar in these studies.

Iqbal et al. compared the dosimetric advantages of between D-IMRT plans and S-IMRT plans (14). They suggested that for the OAR, S-IMRT was able to sustain lower mean and maximum doses compared to D-IMRT. They reported that this dose reduction in critical organs without compromising the dose in target volume could lead to additional clinical advantages. This study is an attempt to-evaluate the impacts of the number of ILs of S-IMRT and D-IMRT planned with conventional MLC. S-IMRT plans resulted in dose reduction to all the

OARs. S-IMRT plans with different ILs showed an overall reduction in OARs compare to D-IMRT plans.

CONCLUCSION

D-IMRT and S-IMRT have similar results related to the PTV coverage. D-IMRT technique slightly increased the OARs dose to normal healthy tissues when compared to S-IMRT. This results showed that S-IMRT has a dosimetric advantage in IMRT treatment plans. Over all, it is suggested that S-IMRT technique is better than D-IMRT technique.

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CHAPTER 12

HOMOCYSTEINE AS A RISK FACTOR FOR CARDIOVASCULAR DISEASES

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1. INTRODUCTION

Cardiovasculer disease (CVD) is the leading cause of death in the world, and one third of deaths occur due to these diseases (1). CVD causes more than 600,000 deaths annually in the United States (2). The main cause of mortality in CVD is ischemic heart disease (IHD). Atherosclerosis is characterized by narrowing or blockage of the coronary arteries that supply the body's nutrients and oxygen to the heart. Atherosclerosis is a complicated inflammatory process and causes important vascular diseases such as cerebrovascular and peripheral arterial diseases, especially in the coronary arteries. Vascular endothelial damage or endothelial dysfunction is the primary step in the development of atherosclerosis. Increased and modified LDL levels, increased free oxygen radicals, hypertension, diabetes mellitus, genetic differences, increased plasma homocysteine concentrations; are possible causes of endothelial dysfunction leading to atherosclerosis. Hyperlipidemia, smoking, obesity, excessive stress, lack of physical activity; are preventable risk factors for atherosclerosis. Among the most emphasized factors are hyperlipidemias. There is an important link between nutrition and cardiovascular diseases, and especially unsaturated fatty acids such as omega-3 and omega-6 reduce the risk of atherosclerosis (3,4). Coronary angiography plays an important role in the diagnosis of coronary artery disease (5). Factors such as smoking, hypertension, hypercholesterolemia, diabetes mellitus are classic risk factors for CVD. It forms the basis of studies that suggest that factors other than those known may have an effect on the etiology of CVD, which

occurs in patients who do not have these risk factors or who are younger than the usual age group. One of the best known factors is hyperhomocysteinemia. Epidemiological studies show that moderately increased Hcy levels are a risk factor for peripheral vascular, atheromatous cerebravascular diseases (6,7) and thrombotic vascular diseases (8).

Increased Hcy levels; It is an important risk factor for CVD and venous thrombotic diseases, and studies supporting that Hcy level is also associated with the amount, prevalence and severity of coronary atherosclerosis are becoming widespread. An increased Hcy concentration is associated with an unfavorable prognosis in patients with CVD, and a gradual correlation was found between Hcy concentration and overall mortality (9-11). High Hcy levels also negatively affect the prognosis in the long-term in patients with acute coronary syndrome (12). Although the mechanism of Hcy in atherosclerosis is not fully explained, it is thought to be caused by multifactorial endothelial damage and endothelial dysfunction (13,14). Hcy also disrupts nitric oxide (NO) synthesis and bioavailability, causing endothelial dysfunction and exacerbation of existing atherosclerosis. NO plays a critical role as a regulatory mediator in angiogenesis. Angiogenesis is inhibited when NO formation is inhibited. In recent studies, it has been shown that hyperhomocysteinemia suppresses endothelial cell proliferation involved in angiogenesis in vivo and in vitro, and weakens angiogenesis in vivo, therefore it is defined as an antiangiogenic factor (15-17). It accelerates atherosclerosis by increasing Hcy, XII and V

prothrombotic factors and decreasing antithrombotic factors together with endothelial-derived nitric oxide. Hyperhomocysteinemia also includes direct endothelial cell damage leading to hyperplasia of smooth muscle cells, contributing to occlusion or narrowing of the vessels. Increased plasma Hcy levels contribute to cardiac morbidity and are positively associated with hypertension (18). Aspirin resistance also increases with high Hcy levels in the blood (19). Therefore, Hcy was considered as an independent risk factor contributing to CAD and the Framingham risk score was contested (20). Low levels of vitamin B12 and high serum Hcy concentration have been associated with coronary artery disease in Asians and have been investigated in two different studies in the Indian population (21,22). Hcy is a modifiable risk factor and folic acid supplementation improves endothelial dysfunction caused by high serum Hcy concentration (23).

2. HOMOCYSTEİNE METABOLİSM

Homocysteine; It is an essential amino acid that cannot be obtained from the diet and has an important role in thiol junctional metabolism. It is a sulfur-containing non-protein amino acid formed by the demethylation of methionine. Methionine taken with food enters the methionine cycle, which is related to the folate cycle, while losing a methyl group, it turns into S-adenosyl methionine (SAM) with the ATP that enters its structure. The first step in Hcy synthesis is the formation of SAM. The methyl group of SAM is cleaved by DNA methyltransferase to form S-adenosyl homocysteine (SAH). In this

intermediate, it loses adenosine via the S-adenosyl Hcy hydrolase enzyme to form Hcy (24, 25). (Figure 1).

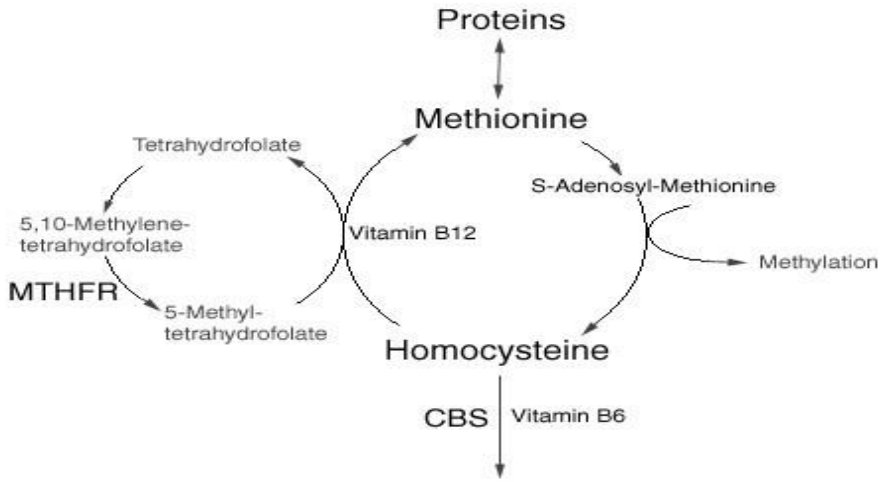

Figure 1. Homocysteine metabolism (25)

According to methionine levels, Hcy; It is metabolized in two ways by participating in one of the transsulfuration or remethylation pathways (25). In cases where hcy protein intake is reduced, it is metabolized by one of two pathways of remethylation. In the liver, most of the Hcy is remethylated by the enzyme betaine-homocysteine methyltransferase (BHMT), using betaine as a methyl source. In most other tissues, Hcy is converted to methionine by taking a methyl group from 5-methyl tetrahydrofolate. This reaction is catalyzed by vitamin B12 dependent methionine synthase (MS). The activity of the enzyme methylene tetrahydrofolate reductase (MTHFR), which forms 5-methyl tetrahydrofolate, which is the substrate of the MS enzyme, is also important in the clearance of Hcy from plasma (25). Hcy also enters the catabolic transsulfuration pathway when there is an excess of

methionine or when cysteine synthesis is required. In this pathway, Hcy combines with serine and is irreversibly converted to cystathionine via the vitamin B6-dependent cystathionine beta synthase (CBS) enzyme. Cystathionine is also converted to cysteine by vitamin B6-dependent cystathionase. The resulting cysteine is converted to inorganic sulfate and excreted in the urine (25,26). As can be seen, the plasma Hcy level may cause a decrease in the activity of certain enzymes or an increase as a result of low dietary intake of vitamins used as cofactors.

The vascular toxicity of hyperhomocystenemia has been fully established. However, there is limited evidence for the effect of hyperhomocystenemia on coronary artery disease in the younger population in the absence of the cumulative and synergistic effect of traditional strong risk factors. Hyperhomocysteinemia results from impaired Hcy metabolism. Severe hyperhomocysteinemia results from rare genetic disorders that result in deficiencies in cystathionine beta-synthase (CBS), MTHFR or enzymes involved in methyl cobalamin synthesis and Hcy methylation.

Increases in Hcy levels typically result from either genetic defects in enzymes involved in Hcy metabolism or dietary deficient intake of vitamin cofactors. It results from a rare congenital anomaly manifested by severe hyperhomocysteinemia and homocystinuria. The most common cause of hyperhomocysteinemia among genetic causes is Cystathionine beta-synthase deficiency (26,27). In the homozygous form of this disease, which is called congenital homocystinuria,

plasma Hcy concentration reaches up to 400 µmol/L at fasting. Although homozygous inheritance is rare, clinical findings such as skeletal disorders, mental retardation, ectopic lens, thromboembolism and severe premature atherosclerosis are encountered in this type. Atherothrombotic complications are frequently seen in homozygotes, especially in young males, and their mortality is high. In heterozygotes, the clinical findings are less pronounced, but the risk of increased vascular events is not obvious. Plasma Hcy levels are generally around 20-40 umol/L. Homozygous deficiency of N5-N10 methylenetetrahydrofolate reductase, which is involved in the vitamin B12-dependent remethylation pathway, also causes severe hyperhomocysteinemia (28). Patients with this type of deficiency have a worse prognosis than those with cystathionine beta-synthetase deficiency, in part due to the lack of effective treatment (29). Such mutations were found to be positive in 38% of the French and 15% of the Canadians and were reported to be the most common cause of moderate plasma Hcy increase (30). Although very common, it is not thought to be an independent risk factor for atherothrombotic disease. Other remethylation cycle disorders related to hyperhomocysteinemia are methionine synthetase deficiency and vitamin B12 metabolism disorders that impair methionine synthetase activity.

The low amount of vitamin cofactors (B6 and B12, vitamins, folate) required for Hcy metabolism in the foods taken may cause hyperhomocysteinemia. Significantly elevated Hcy concentrations have also been observed in dietary deficiency of essential vitamin B12 cofactor and folate cosubstrate (31). It has been reported that there is a

negative correlation between serum folate, vitamin B12 and vitamin B6 concentrations and plasma Hcy concentrations in normal healthy individuals (31). Some studies have also reported a correlation between folic acid deficiency and high plasma Hcy concentration, which is considered a risk factor for various multifactorial diseases (32). Because the causes of elevated homocysteinemia vary, the main appropriate strategies to combat hyperhomocysteinemia, synthetic folic acid supplementation or dietary supplementation, are important (33, 34). However, human studies using natural folates-enriched food matrices to manage hyperhomocysteinemia have been reported as a promising starting point for translation. By adding 0.5 mg of folic acid to the daily diet, total Hcy levels can be reduced by 25% (35, 36). Because the causes of elevated homocysteinemia vary, it has been shown that the main appropriate strategies to combat hyperhomocysteinemia, synthetic folic acid supplementation or dietary supplementation, are important (33, 34). However, human studies using natural folates-enriched food matrices to manage hyperhomocysteinemia have been reported as a promising starting point for translation. By adding 0.5 mg of folic acid to the daily diet, total Hcy levels can be reduced by 25% (35, 36).

CONCLUSION

High serum Hcy concentration increases the risk of CVD. Hcy inhibits endothelial cell proliferation, which plays an important role in the regulation of angiogenesis by causing damage to the vascular endothelial structure. The development of vascular collaterals in the

human heart mainly occurs with the co-occurrence of arteriogenic/angiogenic type adaptations. Collateral circulation is important in terms of being a potential alternative source that occurs and meets the need in cases where there is insufficient blood flow in the coronary vessels to meet the heart's needs.

As it is a modifiable risk factor, maintaining plasma Hcy at normal levels will reduce cardiovascular mortality.

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CHAPTER 13

ENZYMES AND THEIR APPLICATIONS IN MEDICAL SCIENCES

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1. INTRODUCTION

Human body is composed of various types of cells, tissues, and different complex organs. Countless chemical reactions get carried out in a given time in every living organism. For effective functioning in every living organism, the body releases several chemicals which perform the function of accelerating biological processes like digestion, respiration, excretion, and some other metabolic pathways.

The cell is a basic building block of the living system. Under the normal physiological conditions (constant pressure, nearly neutral pH, and temperature of about 20-40℃) many of these reactions might function gently or cannot happen. The specific chemicals that allows these reactions to be carried out with high speed and under natural conditions which are convenient for life. A catalyst is able to accelerate the chemical reaction without getting involved in the final product. In biological media, macromolecules known as enzymes act as catalysts [1]. Enzymes are functional proteins that process cellular metabolism. They affect by catalyzing the reaction, also known as biological catalyst (bio-catalyst), and can be used to perform various biochemical reactions which are desirable for normal functioning, proliferation, and growth of the living system. Cells can effectively utilize this bio-catalyst known as enzymes. These enzymes have high level of specificity towards their substrate and remarkable catalytic power which makes them suitable for chemical reactions in a living body [2]. Hence, enzymes are pivotal in all the living entities that govern all the biochemical reactions performed inside the living body.

"Enzymes can be described as a biological polymer that helps in catalyzing various biochemical reactions"

Enzymes are synthesized to encounter the metabolic requirements of body tissues and are not always tissue specific. More than one organ/tissue can synthesize one or more enzymes [3, 4]. This enzyme helps in transforming exogenous substances, to obtain basic materials and energy, which is carried out with the diet, for the synthesis of endogenous molecules.

The set of enzymes carry out various metabolic processes and biochemical reactions in the cell which are necessary to sustain life [5]. Enzymes are responsible for catalyzing almost all the reactions in living organism because they are specific and act as an efficient bio-catalyst which reacts with different substrates in biochemical reactions which includes photosynthesis, phosphorylation, respiration, dehydration, hydrolysis, movement growth, excretion of toxic chemicals from the liver and other parts. Enzymes are proteins, to get active they should possess a correct structure and get very easily affected by pressure, temperature, pH, and metal ion.

2. ENZYME MARKET SCENARIO

The global healthcare enzyme market size is estimated at USD 2.4 billion in 2019 and is predicted to rise at a CAGR (compound annual growth rate) of about 6.2% from 2020 to 2029 [6]. The increasing prevalence of diseases related to metabolism has been observed in recent times. Therefore a relevant understanding of enzyme

abnormalities has led to a rise in the use of enzymes in clinical examinations as disease markers.

Laboratories hold the maximum revenue of 44.8% in the global healthcare enzyme market in 2019 [6]. This is because of the ample usage of enzymatic products in the research operations. Research is getting targeted towards exploring the potential of healthcare specialty enzymes products due to which laboratories are keen on adopting enzyme products for disease diagnosis such as myocardial infarction, infectious diseases, and inflammatory disorders.

Hospitals are largely adopting enzyme-based diagnostic tests, especially for the treatment of oncology and cardiac patients. Changing levels of (LDH) Lactate Dehydrogenase in the blood is observed for detecting the rise of cardiovascular diseases; as these enzymes are found in the heart and skeletal muscles [6].

Insights associated with the market prediction of the most widely used enzymes such as lipases, carbohydrase, proteases and their marketplace for aid product and pharmaceutical sectors area unit bestowed here. Lipase the main growth of the lipase market can be in the health management sector for the treatment of deficiencies of micronutrients like obesity. As fats get broken down into fatty acids and glycerols under normal physiological conditions, their demand is supposed to increase in the healthcare sector as an aid for weight loss [7]. Proteases the proteases market can be of major importance to the health industry because of numerous benefits offered by enzymes, like

curing skin burns, stomach ulcers, and preventing inflammatory diseases.

Specialty healthcare enzymes have noticed major demand over clinical applications due to rise in the use of enzymatic assays and techniques. For example, β-galactosidase, peroxidase, and alkaline enzymes are extensively incorporated within the Enzyme-Linked Immunosorbent Assay (ELISA) protocols [5]. The vast demand for ELISA in communicable diseases digestion is about to spice up the expansion of the marketplace for tending specialty enzymes. These are being incorporated within the detection of analytes like hormones, drugs, bodily fluid parts, and oncofetal proteins.

Highly efficient reactions are catalyzed by the enzymes. Enzymes can also function outside the living organisms (or the cells), hence can be utilized as free natural forms or as in immobilized forms [7]. They are used in various applications such as medical diagnosis, therapeutics, and synthesis of pharmaceutical products such as drugs.

3. STRUCTURE OF ENZYMES

The complete knowledge of enzyme mechanisms needs a correlation of pieces of information like the structure of enzymes, their dynamic natures, and other information.

Structural enzymology is a study concerned with the molecular structures of enzymes, how they acquire their unique catalytic activity, and how the changes in their structure affect their functioning [8]. The structural arrangements of enzyme proteins such as Primary,

Secondary, Tertiary, and Quarternary structures are of vital importance for their catalytic activity. These structures are represented in Figure 1, 2 and 3. The primary structural configuration and action of enzyme catalysis are determined by the linear chain of amino acids which are linked by peptide bonds [2]. This bond possesses double-bound characteristics and is always in trans-conformation. The localized folding of the polypeptide sequence is called Secondary structure like α-helices, β-sheets, or irregular coils. The further folding is known as Tertiary structure, this tertiary structure may consist of a single domain or a few multiple domains in appropriate adaption. Many proteins are oligomers of subunits, a Quaternary structure is the agglomeration of these several subunits chains.

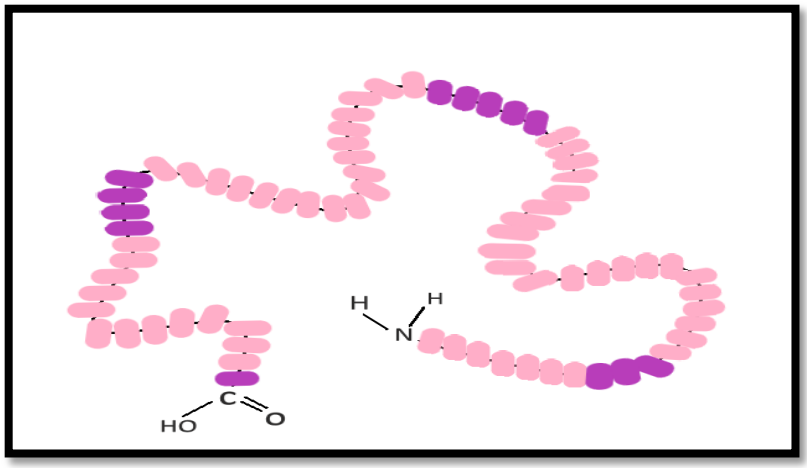

Figure 1: Primary structure of the enzyme

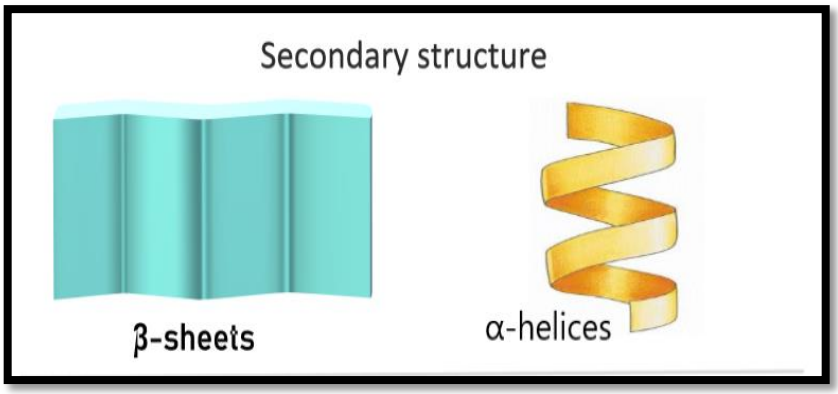

Figure 2: Secondary structure of enzyme

Enzymes are natural large protein molecules with molecular weights ranging from about 12,000 to greater than 10,00,000 Dalton, which can permit reactions to get carried out in the mild conditions that can be tolerated by our living body [2]. Changes in pH and temperature create a great impact on the intra- and intermolecular bonds that attach the protein molecules to create secondary and tertiary structures.

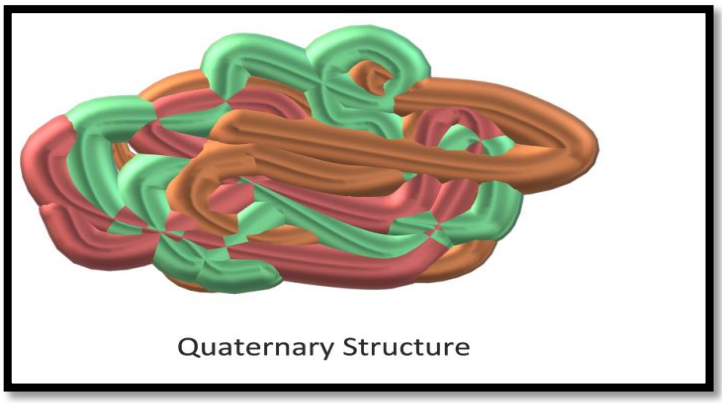

Figure 3: Quaternary structure of enzyme

Enzyme structure and its active site determine enzyme specificity [5]. The active site is the part of an enzyme where the substrate binds. The shape of an enzyme changes slightly here by fitting tightly with the substrate and forming the enzyme-substrate complex [9]. This enzyme-substrate complex enables the enzyme to demonstrate its specificity in various catalytic activities.

The active site is the only part where the substrate gets bind to enzyme molecule. Therefore the remaining protein molecules perform the function of stabilizing the active site and providing the relevant environment for the interaction of substrate molecule to the active site.

Every enzyme has a globular protein part known as apoenzyme and a non-protein part called the cofactor. For an activity to be performed enzymes require a cofactor, this cofactor and apoenzyme together are known as a holoenzyme (Figure 4).

Apoenzyme + Coenzyme ⇔ Holoenzyme

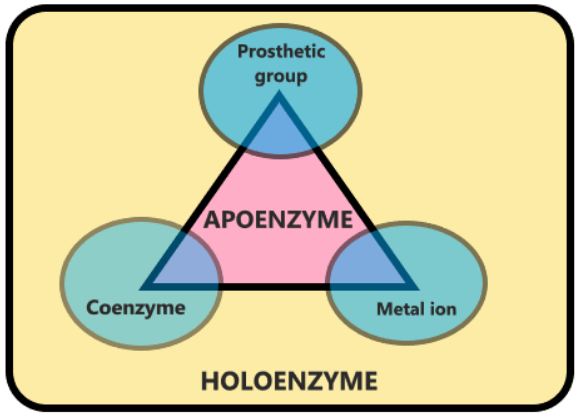

Figure 4: Holoenzyme

3.1. Classification of enzymes

Enzymes are classified by adding –ase as the suffix to the unit substrate modified by them (i.e tyrosinase, lipase, and urease), or by, the reaction catalyzed by them (i.e decarboxylase, hydrolases) [10]. Some of the enzymes are identified by random names (trypsin and pepsin).

Enzymes classification is based on the reaction they catalyze. Enzyme classification (EC) system consist of 6 major groups of enzymes which are as follows:

Oxidoreductases:- These enzymes are associated with coenzyme and catalyze redox reactions. They include oxygenase, oxidases, dehydrogenases, and peroxidases.

Transferases:- These enzymes perform the function of catalyzing the transfer of the atoms (methyl, acyl, carbonyl, carboxyl, phosphoryl, and glycosyl) to acceptor compound by the donor substrate. Example:- Aspartate aminotransferase.

Hydrolases:- These enzymes catalyze the breakdown of ester (O-P, C-S, C-N, and C-O) bonds by introducing them to water. Example:- l-arginine amidino hydrolase, is an enzyme which performs the function of catalyzing the hydrolysis of arginine to form urea as the product. Another example is the ribonuclease which catalyzes the hydrolysis of bonds between nucleotides in RNA (ribonucleic acid).

Lyases:- These enzymes catalyze the breakdown of non-peptide (C-S, C-C, and C-N) bonds of the substrate. Here the breakdown process is

not similar to hydrolysis. Here some enzymes of this class separate groups from the substrate molecule to form double bonds or cycles, whereas some enzymes add groups to the double bonds. When these enzymes perform reverse reactions, they are named synthase. Example:- The cleavage of fructose-1, 6-bisphosphate is catalyzed by the enzyme aldolase to produce two trioses phosphate.

Isomerases:- Isomers of any type (geometric, optical, or positional) are interconverted by this class of enzyme. Example:- phosphoglucoisomerase is an enzyme that performs the catalysis of conversion of glucose-6-phosphate into fructose-6-phosphate.

Ligases:- These enzymes catalyze the binding activity of two molecules in operation that is coupled to the hydrolysis of a high-energy bond of a nucleotide triphosphate. These enzymes are commonly designated as synthetases. Example:- glutamine synthetase is an enzyme that performs the catalytic reaction to form glutamine by using ammonia and glutamic acid. Here the hydrolysis of ATP provides the energy for the synthesis.

Isoenzymes are different types of enzymes produced by various tissues. Although all forms of particular isoenzymes catalyze the same reaction, their structures are slightly different and their location within body tissues may vary [11]. Example:- The enzyme LDH (lactate dehydrogenase) can find useful in the diagnosis of various diseases by observing the serum levels of LDH. Diseases such as acute liver diseases, muscular disease (muscular dystrophy), heart failure, and anemias including the rupture of red blood cells.

Cofactors:- These are the non-proteinous structures that bind with enzymes to perform the essential functioning of an enzyme. Enzyme and cofactor together are known as holoenzymes, whereas enzymes lacking cofactors are called apoenzymes [1]. There are 3 types of cofactors enzymes binds with are as follows:

- Coenzyme:- A coenzyme gets attached to an enzyme only while performing catalysis, all other times it is detached from the enzyme. Example:- NAD^+
- Prosthetic groups:- These are the cofactors that are always firmly bound to an enzyme. Example:- heme, fad, biotin.
- Metal Ions:- For the catalysis of certain enzymes, a metal ion is required at the active site to create coordinate bonds. Zn^{2+} is a metal ion cofactor used by numerous enzymes [1].

3.2. Mechanism of enzyme action

For any reaction to take place two molecules collide with the right orientation and an adequate amount of energy. This energy is known as activation energy [5]. The mechanism of enzyme action relay on the capability of enzymes which accelerates the rate of reactions and decreases the activation energy. When the reaction occurs, the substrate (S) and the enzyme (E) together bind with each other and forms an (ES) enzyme-substrate complex [4]. When the reaction is finished it provides us with the product and the enzyme remains unchanged and can be further used to bind with another substrate.

The surface of an enzyme contains a small region where only the specific substrates binds known as an active site. When the substrate is

attached to the active site with the help of intermolecular forces it forms an ES (enzyme-substrate) complex [9]. As the complex is formed the reaction of conversion of substrate to product is carried out. After the completion of the conversion reaction, the product releases the active site setting the enzyme free to bind with another substrate molecule.

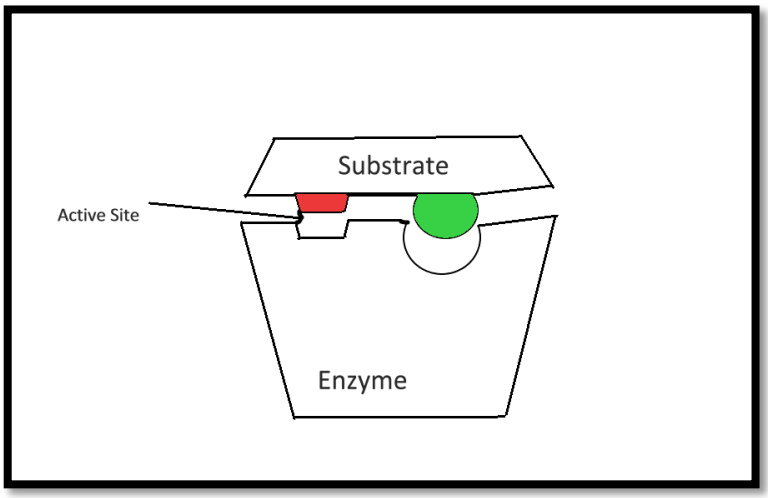

Figure 5: Active site of an enzyme where substrate binds

The enzyme specificity completely depends on the active site of an enzyme and the nature of the substrate. This hypothesis was first proposed by Emil Fischer a German chemist in 1894, and is known as Fischer's "lock and key model" (Figure 5). This theory explains the action of enzyme specificity. The modification of the lock and key theory was further known as Induced-Fit theory which explains that enzymes have flexible conformation that gets adapted after the substrate is attached to the active site.

Enzyme kinetics: the speed of reaction catalyzed by the enzymes is studied with the help of enzyme kinetics. This contains some mathematical equations. This theory of enzyme kinetics is simple and logical, and it creates a great understanding of the enzyme action and activity [9]. The reactions catalyzed by the enzymes rise towards the higher level of concentration which is known as Vmax which indicates the enzymes reached their maximum point.

The relationship between enzyme or substrate concentration and the rate of the reaction catalyzed by the enzyme is described in the form of an equation which is as follows:

$$y = \frac{a \times x}{x + b}$$

In the study of enzymology, this equation is referred to as the Michaelis constant (Km), which is defined as the substrate concentration that gives half-maximal velocity [9]. Hence the Michaelis-Menten equation also can be represented as follows, with k_1, $k_{-1,}$ and k_2 being the rate constant of the three individual reaction steps:

$$\text{Initial rate of reaction } (v_0) = \frac{\text{Vmax} \times \text{Substrate concentration}}{\text{Substrate concentration+Km}}$$

The K_m value provide us the information about several important aspects of particular enzymes [9]

- An enzyme with a low Km value relative to the physiological concentration of substrate will probably always be saturated with substrate, and will therefore act at a constant rate, in any

case if there is change in the concentration of substrate within the physical range [9].

- An enzyme with a high Km value relative to the physiological concentration of substrate will not be saturated with substrate, and its activity will therefore vary according to the concentration of substrate, so the rate of formation of the product will depend on the availability of substrate [9].
- If an enzyme acts on several substrates, the substrate with the lowest Km value is frequently assumed to be that enzymes 'natural' substrate, although this may not be true in all cases [9].
- If two enzymes (with similar Vmax) in different metabolic pathways compete for the same substrate, and know the Km values for the two enzymes we can predict the relative activity of the two pathways [9]. Essentially the pathway that has the enzyme with the lower Km value is likely to be the 'preferred pathway', and more substrate will flow through that pathway under specific conditions. For example, phosphofructokinase (PFK) is the enzyme that catalyzes the first committed step in the glycolytic pathway, which generates energy in the form of ATP for the cell, whereas glucose-1-phosphate uridylyltransferase (GUT) is an enzyme early in the pathway leading to the synthesis of glycogen (an energy storage molecule) [9]. Both enzymes use hexose monophosphates as substrates, but the Km of PFK for its substrate is lower than that of the GUT for its substrate. Thus at lower cellular hexose phosphate concentrations, PFK will be active and GUT will be

largely inactive. At higher hexose phosphate concentrations both pathways will be active [9]. This means that the cells only store glycogen in times of plenty, and always give preference to the pathway of ATP production, which is the more essential function [9].

There are about two types of enzymes, one of the enzymes (Figure 6) helps in joining specific substrate molecules together and forms a new product, and the another type of enzyme (Figure 7) helps in the breakdown of a substrate molecule into separate molecules [11].

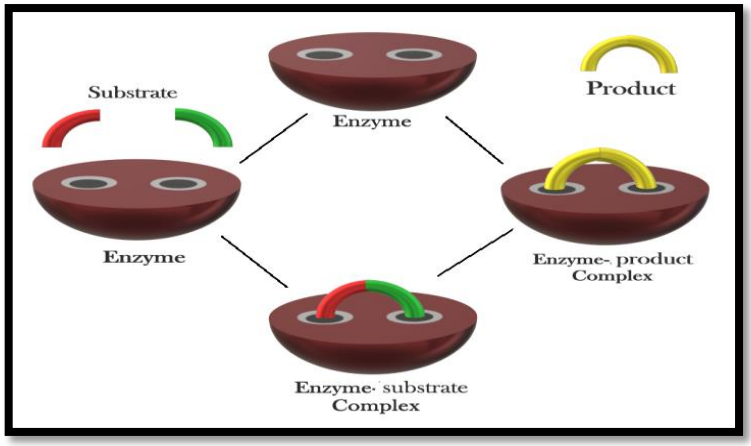

Figure 6: Type I enzyme

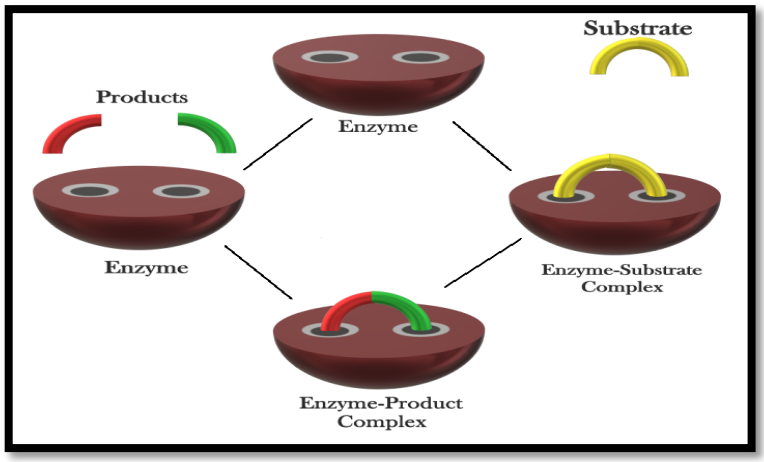

Figure 7: Type II enzyme

Enzymes carry out various functions in our living body such as signal transduction, they perform break down of larger molecules into smaller molecules, movement of ions across the plasma membrane, helps in generating energy as the enzyme ATP synthase is involved in the synthesis of energy.

3.3. PROPERTIES OF ENZYMES

Enzymes are extremely efficient catalysts that are produced by the living system, within the natural conditions of mild pH, pressure, and temperatures they carry out numerous chemical and biological reactions. And can raise the rate of reaction by 10-20 times when compared to uncatalyzed reactions [5]. They can also be defined as colloidal, soluble, and organic catalysts that are produced within the living cell but are capable of performing their catalytic activity independently outside the cell. Enzymes have three major properties in which they are well suited to their roles: 1) They consist of

enormous catalytic power, 2) The reactions catalyzed by them are highly specific and 3) Their activity as catalysts can be regulated [11].

Enzymes are the long chain of polymer units that can be folded into complex structures. As a result, they can be more specific than inorganic catalysts in which substrates they recognize and bind to [11]. Enzymes can be regulated in any way compared to an inflexible, rigid, metallic inorganic catalyst, as enzymes are found to be flexible. Enzyme specificity depends upon the structure and flexibility of its active site. The biochemical nature of the cell also can be indicated by the effects of enzymes on cellular metabolites, this can also be explained by the enzyme's property of flexibility [15]. Allosteric regulation is the phenomenon where the catalytic rate of reaction changes due to a structural change in the enzyme which occurs when enzymes bind to such metabolites. Enzyme provides an alternative reaction pathway of lower activation energy which speeds up the reactions and lowers the activation energy of the reactions which is a required amount of energy needed for the reaction to occur [8]. Enzymes are biodegradable, non-toxic, have amazing catalytic power, and their high level of specificity for their substrate makes them suitable for various biological reactions.

The type of reaction catalyzed by the enzymes are very specific [7]. The enzyme with absolute specificity catalyzes the reaction of one and only one substance, the enzymes with relative specificity catalyze the reaction of structurally related substances, and the enzymes with

stereochemical specificity catalyze the reaction of only one of two possible enantiomers [8].

Enzymes are reusable as they are not utilized in the reaction. Once the substrate and the active site of the enzyme binds together they perform catalysis of the reaction. Further, the enzyme is released and remains unchanged, and is ready to be used for another reaction.

3.4 Factors affecting enzyme activity

The activity of enzymes can be affected by some of the factors, such as pH, temperature, and substrate or enzyme concentrations. Every enzyme has an optimum range of pH [16]. Changes in the pH values can lead to low down the activity of an enzyme, and also can cause denaturation of the enzyme. As we know increasing the temperature generally speeds up the reaction, and decreasing the temperatures reduces the reaction rate. Upon heating at extremely high temperatures structure of enzymes gets to denature and may stop working [17]. Increasing the concentrations of both enzymes and substrate increases the speed of reaction, as long as there are both of them available to bind with each other they speed up the reaction as the binding gets complete and no enzyme or substrate is present to bind further, the reaction stops speeding up and continues to work at their maximum rate.

4. THE HISTORICAL IMPORTANCE OF ENZYMES AND THEIR SOURCES

Berzelius has described the catalytic nature of fermentation in late 1837. Fermentation is a process initiated by the living body, this concept was described by Louis Pasteur in the 1850s where he also described the ferments which were structures close to yeast that catalyzed the fermentation of sugar into alcohol. Later these ferments were termed enzymes. Emul Fischer reported the systematic study on enzymes by introducing the 'Lock and Key' model on enzyme specificity [1]. Further advancement in the history of enzymes was brought up by Edward Buchner by isolating soluble active forms of an enzyme from yeast cells in 1897. James Sumner extracted urease from jack beans in 1926 [18]. Later, in 1953 Koshland introduced the hypothesis of 'Induced fit' model [12]. Johnson, Phillips, and North in 1965 successfully identified the 3-D structure of lysozyme [13]. In the subsequent year's enzyme research developed rapidly. The important developments were the detection of various biochemical processes like coagulation, endocrine function, digestion, and various metabolic process required for the living organism. Exhaustive research was performed on enzyme-catalyzed reactions and for the enzymes that participate in cellular metabolism. At present, 2000 different enzymes have been recognized, each of which catalyzes a different reaction [2].

Recently, the focus is directed towards the application of enzymes. The properties of enzymes such as high specificity and their efficiency

increase their commercial value and applications towards clinical medicines.

4.1. Sources of enzymes

Enzymes are found everywhere from the bottom of the ocean to your backyard, and even in our bodies [19]. Plant-based enzymes are found in plants like papain is an enzyme present in papaya. The well-known enzymes found in plants are protease, amylase, lipase, and cellulose. Protease performs the function of breaking up the proteins found in eggs, meat, cheese, and fish [20]. Amylase helps the human body with the absorption of carbohydrates and starch. Lipase helps in fat digestion. Cellulose is a very important plant-based source as it is not naturally produced by the human body.

Microorganisms-based enzymes are further categorized into three different source types, namely yeast, bacterial, and fungal. Phenol oxidases, hydrolases, and esterases are mushroom-based enzymes. Bacterial enzymes are sourced generally from *Bacillus* species of bacteria. Application of these enzymes is found in various pharmaceutical and food industries [21]. Fungal enzymes are prominent due to their increasing utilization in various industries. Microbial enzymes have some obvious advantages as they can be produced in large quantities at less cost. Production of microbial enzymes remains unaffected by the seasonal changes. Enzymes must be stable to provide high yield for various applications. As different types of microorganisms can survive in different extreme conditions,

accordingly, enzymes produced by them must be stable in different extreme conditions [19].

5. APPLICATIONS OF ENZYMES IN MEDICAL SCIENCE

5.1. CLINICAL ENZYMOLOGY

Clinical enzymology introduces us to the measurements of enzyme activity for the applications like diagnosis and treatment of various diseases [22]. As any malfunction such as mutation, deletion, over-production, or under-production of enzymes may lead to a genetic disorder generally known as inborn errors of metabolism. Phenylketonuria is the most common type example of a mutation in an enzyme phenylalanine hydroxylase which performs the function of catalyzing the degradation of phenylalanine [23]. This deficiency can lead to the build-up of phenylalanine and can result in mental retardation if remains untreated.

5.2. ENZYMES IN MEDICAL DIAGNOSIS

Enzymes have found significant roles in clinical laboratories for the diagnosis and treatment of various diseases [4]. The enzymatic assay play a significant role in diagnosis of various diseases depending on where they are found in the body because a minute change in the levels of the enzyme concentrations can be measured easily by these enzymatic assays.

Enzymes of the heart, kidney, liver, skeletal muscles, etc. escape into the blood due to some disorders (Table 1). Quantifying the number of equivalent proteins for the presence of high or low levels into the

blood specifies the particular disorder. Example: - For the diagnosis of muscle injury or weakness levels of creatine kinase are measured [19].

Table 1: Important enzymes in the diagnosis [4]

Enzymes	Disorders
GPT, GOT, ALP, GGT	Liver Function Tests
Troponins, CK-MB	Cardiac Function Tests
Amylase, Lipase	Pancreatic Enzymes
LDH, CK	Muscle Enzymes
ALP, ACP	Bone Enzymes

Diseases usually result in average or substantial tissue damage depending on how serious is the disease. These conditions are commonly related with the release of specific enzymes into circulation of diseased organ tissue and this causes increased activity of these enzymes in body fluids [4]. Hence measuring the activity of enzymes in plasma/serum and other fluids of the body have been employed in disease diagnosis. Various enzymes used in diagnosis are mentioned.

There are various enzymes which performs important functions in the diagnosis of various diseases/disorders which are as follows:

Lipase: Lipase is an enzyme which performs the function of breaking down fats from the food to get used up by the human body. They are found in the pancreas, stomach, and mouth. Various skin disorders and diseases like pancreatitis are diagnosed by the help of this lipase enzyme [24].

Lactate dehydrogenase (LDH): LDH enzyme helps in the process of converting sugar into energy for the cells. This enzymes is majorly found throughout the human body in various organs such as pancreas, liver, kidney, heart, blood cells, and skeletal muscles. Hence observing the levels of this enzyme is very important in the diagnosis of many liver diseases, kidney diseases, and various heart problems [33].

Leukocyte esterase: This enzyme used in the diagnosis of Periprosthetic joint infections [27].

Aspartate transaminase (AST): Dental disorder such as Periodontal disease is a general inflammatory disease of the oral cavity. Higher levels of AST in GCF (gingival crevicular fluid) of a diseased site can serve as the potential biomarkers for the diagnosis of periodontal disease when compared to the levels of AST in saliva [25].

Glucose-6-phosphate dehydrogenase: This enzyme used in the diagnosis of Gastric cancer [35].

Alkaline phosphatase (ALP): Alkaline phosphatase functioning under alkaline pH, is classified under hydrolases and removes phosphate groups from nucleotides and proteins. An increase in the level of ALP in serum indicates the rise of osteoblastic activity or rheumatoid arthritis when there is active bone formation. Other physiological conditions such as hyperthyroidism, hyperparathyroidism, osteomalacia, and rickets result in increased levels of ALP [36]. Low ALP activities are rarely common and are related to a genetic

condition or nutritional deficiency [37]. Hypophosphatasia is a disorder characterized by reduced levels of serum ALP and is rarely found. Resulting with skeletal abnormality and abnormal phosphorylated metabolites.

Muscle diseases include myopathies (diseases of muscle fiber) or neurogenic disorders (diseases of muscle nerves). Damage to the muscle can be due to physical trauma, the extensive exercise of muscles, drugs, microbial infection, inflammatory diseases, or it may be genetically predisposed. In neurogenic diseases, CPK (creatine kinase) is released occasionally [16]. Whereas in myopathies ALD, LD, GPT, and GOT levels are increased. Thus by performing the enzymatic assay for the observation of these enzyme levels in the serum/blood can help in the diagnosis of various diseases. Many enzymes are characteristically used by clinical laboratory for diagnosis of various diseases. Pancreas, red blood cells, liver, heart, brain, prostate gland, and many of the endocrine glands contains highly specific markers which activates the enzymes [38].

The enzymes in the analysis are generally the soluble enzyme “kits”, found to be very useful in clinical biochemistry for the quantification of levels of blood glucose by observing catalase, glucose oxidase and by using cholesterol oxidase observe serum cholesterol levels.

5.3. ENZYMES APPLICATIONS IN PHARMACEUTICAL INDUSTRY

The enzymes also show vast applications in the field of pharmaceuticals, this chapter focuses on the ability of biocatalysts in the manufacturing of drugs, healthcare supplements, API, and enzyme-based therapy for the treatment of numerous diseases. When we consider drugs, enzymes shows two important characteristics which make them different from various other types of drugs [37]. First, enzymes bind on their targets substrate and act with great specificity and affinity. Second, enzymes convert multiple substrate molecules into specific products and shows the catalytic activity [39]. These characteristic features of enzymes make them useful in the pharmaceutical industry for the treatment of a wide range of disorders. Several drugs and special pharmaceutical formulations are made up of APIs which can be synthesized with the help of enzymes as the major component of the manufacturing process.

5.3.1 Enzymes used for synthesis of antimicrobial

Penicillin acylase is the group of enzymes generally produced by fungi, yeast, actinomycetes, and bacteria. Based on their substrate specificity they are divided into three groups: ampicillin acylase, penicillin V acylase, and penicillin G acylase [16]. The use of penicillin acylase as a catalyst for the synthesis of 6-APA took over the traditional chemical synthesis process. As the enzyme used methods for the production of 6-APA results with good yield percentages when compared to the conventional chemical process by

using various hazardous reagents and solvents. Further, some researchers proposed that penicillin V acylase can be a great alternative for the synthesis of 6-APA which has greater stability at lower pH and leads to a higher yield when compared with the process of synthesis by penicillin G acylase [36]. Semisynthetic penicillins result in much better properties compared to penicillin V and G, as they have higher stability, are easy to absorb, and show fewer side effects. The extensive production of semisynthetic antibiotics produced from penicillin depends on the condensation of the β-lactam nucleus and appropriate D-amino acid catalyzed by penicillin acylase. Kinetic enantioselective acylation of azetidinone intermediate is another application of penicillin G acylase which is used for the synthesis of carbacephalosporin antibiotic Loracarbef and anti-platelet agent Xemilofiban [40]. The cephalosporin derived antibiotics are considered to be most important and efficient key drug towards the treatment of various infections and bacterial diseases.

5.3.2 Enzymes used for synthesis of amino acids

Amino acids are also known as the building blocks of life. Due to their chirality, amino acids are very significant and can be used for various biochemical reactions and chemical synthesis processes. l-histidine, l-isoleucine, l-valine, l-methionine, l-leucine, l-lysine, l-phenylalanine, l-threonine, and l-tryptophan are the nine essential amino acids that cannot be synthesized in humans or animals [41]. Enzymes are very important for the production of non-proteinogenic D- amino acid and L- amino acids and proteinogenic which are considered to be the

important active ingredients in the manufacturing of various pharmaceutical drugs, cosmetics, and agrochemical industries [36]. L-methionine, is considered in special diets, which is produced by the enzymatic resolution of the acylase enzyme. L-aspartic acid is another example of amino acid which is aquired by the catalysis of the enzyme aspartase. Further by using aspartate β-decarboxylase enzyme as a catalyst L-Alanine is produced from L-aspartate.

Xemilofiban is an antiplatelet agent synthesized by the enzymatic activity. An enantioselective acylation is catalysed by penicillin G amidohydrolase which resolves the racemic mixture of ethyl 3-amino-5-(trimethylsilyl)-4-pentanoate extracted from E.coli to yield the isomer, which can be utilized as a chiral synthon for the synthesis of Xemilofiban [41].

Enzymes used for the manufacturing of APIs results in various advantages such as improving productivity like shortening the synthesis route by increasing yields; providing greater saving potential by replacing costly resolving agents, and production of less waste; providing a high level of stereo-, regio-, and chemoselectivity; producing fewer byproducts, and reducing impurities of the products.

5.4. ENZYMES IN THERAPEUTICS

Enzyme therapy introduces us with the enzyme applications like treatment of enzyme deficiencies, enzyme disorders and other medical conditions in humans [7]. In the humans enzymes performs the function of food digestion, boosting the immune system, body detoxification, reduction of stress of organs like the pancreas, liver.

Due to the various medical applications enzymes are used as therapeutics in various medical treatments such as treatment of pancreatic insufficiency, metabolic disorders, cystic fibrosis, cancers or tumors [7].

Enzymes are generally used in three cases: 1) to break the internal blood clots. 2) To dissolve the hardening of walls of blood vessels. Example: - serratiopeptidase 3) To dissolve the wound swelling to promote healing [19]. Example: - chymotrypsin, trypsin

5.4.1. Enzymes applications as digestive aids

Numerous enzymes are capable of healing various digestive problems induced by the sugars. People develop symptoms such as diarrhea, bloating, or gas due to the uptake of foods like sprouts, beans, vegetables such as broccoli, cabbage, and so on. The enzyme α-galactosidase is suggested as a digestive aid for such problems. As α-galactosidase performs the function of breaking down the α-galactosidic residues of sugars present in the food, which remains undigested and thus cause discomfort [42].

The other digestive problem faced generally by humans is lactose intolerance. It is the condition in which the human body is unable to digest milk sugar lactose because the body is unable to produce the lactase enzyme in sufficient quantities [40]. The lactose-intolerant people are unable to digest lactose-based products like milk and thus suffer from stomach upset when they consume it. For such lactose intolerant people, various supplements containing lactase such as lactase powders are suggested as an aid. People with weak immunity

are generally suggested with digestive aid such as a mixture of some pancreatic enzymes which includes amylase, lipases, and proteases. [43].

5.4.2. Enzymes used in the treatment of damaged tissues

Various proteolytic enzymes from plants and bacterial sources have been studied for the treatment of burns by the removal of dead skin. Debrase gel dressing is the mixture of various enzymes extracted from the plant source (pineapple), this gel dressing is an agent that easily and specifically removes the eschar tissues from the wounds caused by burns by the enzymatic action, and have received clearance from the US FDA for the treatment of full- and partial-thickness of burns [43]. The (Asparaginas) enzyme originated from C. perfringens shows applications against skin ulcers [51]. The regeneration of the injured spinal cord has been demonstrated using the enzyme chondroitinases, where these enzymes functions by removing the glial scar and then accumulating chondroitin sulfate which exhibits the growth of axon [44]. The proteolytic enzyme (vibrilaseTM) from vibrio proteolyticus shows effective results against the denatured proteins found in burn wounds.

5.4.3. Enzymes used in the treatment of infectious diseases

Lysozyme is an enzyme produced in the human body naturally and acts as a bactericidal enzyme. Studies have shown its activity against HIV and can selectively degrade viral RNA [45] and can be a promising enzyme in treatment of HIV infection, Gastritis, Dyspepsia, and antibiotic. Another example is Chitinases which is an

antimicrobial enzymes, as chitin is found in major quantity in the walls of various pathogens such as fungi and protozoa. Collagenase is an enzyme produced in the fruit (*Ananas comosus*) shows therapeutic applications against many upper respiratory tract diseases. Ribonuclease (RNases) are the large hydrolytic enzymes which function the breaking down of RNA. They are originated by yeast and bacteriophages, and shows applications in therapeutics as antivirals [51].

5.4.4. Enzymes used in the treatment of cancer

Enzymes having therapeutic applications such as treatment of cancer is an emerging field of research [46]. PEG immobilized arginine deaminase has the capability of inhibiting skin cancer and hepatocellular carcinomas which lack arginine due to the deficiency of arginosuccinate synthetase activity. Greater enzyme therapy has been developed by using PEGylated I-asparaginase known as Oncaspar. It has shown much better results in the treatment of acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma [47]. Asparaginase and PEG-asparaginase can be provided as a better alternative to the traditional standards of chemotherapy.

5.4.5. Enzymes used in oral and inhalable therapies

In contrast to the treatments mentioned so far, some diseases do not require an intravenous injection of an enzyme of human origin. Congenital sucrase-isomaltase deficiency (CSID) can be treated with help of enzyme sacrosidase—a β-fructofuranosidefructohydrolase obtained from Saccharomyces cerevisiae and can be taken orally [40].

PKU (Phenylketonuria) is a genetic disorder that is complied with the specialized diet [46]. This disorder is caused due to low phenylalanine hydroxylase activity, which is responsible for the catalysis of the reaction which converts phenylalanine to tyrosine. By the use of PAL (phenylalanine ammonia-lyase) from recombinant yeast the oral treatment is developed as phenylase which functions by degrading the phenylalanine found in the gastrointestinal tract. Inhalable enzyme formulations have showed application in the treatment of (CF) cystic fibrosis. Pulmozyme (Dornase α), a DNase, received one of the fastest approvals by the FDA under the Orphan drug status [47]. Dornase α functions to liquefies the accumulated mucus in the lung. Dornase α used in CF patients reduces the levels of matrix metalloproteinases in the broncho-alveolar lavage fluids which results diminish pulmonary tissue destruction [48].

5.5. ENZYMES IN MEDICAL CLEANING AND DETERGENTS

Enzymatic detergents are specially designed for cleaning reusable medical devices. Studies have proved their potential over non-enzymatic detergents in various applications. Enzymes like trypsin can be used as surface disinfectants [6]. Protease and Lipase are the two enzymes recently used for cleaning medical devices. Proteases perform the role of breaking down protein-rich molecules like blood, whereas lipases focus on fatty oils like adipose tissues [48]. Enzymes like cellulases and amylases are the enzymes used traditionally for

cleaning application which performs the function of breaking down starch and cellulosic polymers.

5.5.1. Enzymes in personal care products

Personal care product is the new immerging field and can be considered as a new area for enzymes. But enzymes can have various applications in this field. Enzymes like lipase and proteinase can be used for contact lens cleaning applications [49]. Hydrogen peroxidase can be used as a disinfectant for contact lenses. Glucose oxidase and glucoamylase have their application in toothpaste which functions as a disinfectant. Enzymes like chitinase have applications in hair and skincare products.

6. CONCLUSION

Enzymes have become a considerable choice in medical sciences because of their functionality and high specificity. Enzymatic processes restored the traditional chemical-based processes for the synthesis of APIs by the synthesis of semi-synthetic pure forms of amino acid and synthesis of antimicrobials by the use of enzymes. Enzymes also show several therapeutic applications such as enzyme therapy used for the treatment of several genetic and metabolic disorders. More recent uses of enzymes are in the treatment of infectious diseases and cancer treatment, where antibiotics are no longer useful. Recent research also shows ample scope for enzymes in the field of medical diagnostics, pharmaceuticals and white biotechnology. In the future, immobilized enzymes can provide more advantages and applications in the field of medical sciences.

7. ACKNOWLEDGMENT

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CHAPTER 14

THE CONCEPT OF ERROR IN HEALTH AND THE TECHNICAL ASPECT OF UNDESIRABLE EVENTS: CURRENT APPROACHES

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INTRODUCTION

The concept of ‘medical error’ in healthcare is a common public health problem all over the world. But no consensus has been reached on the definition of Health error or medical error. In a brief statement, the omission of what should be done has been described as incomplete or incorrect (Rodziewicz Thomas L.). et al, 2021). Even if it is possible to identify the cause of an error, both in the field of Health and in other areas, it is difficult to find a solution to prevent its recurrence, but it is vital. Because trying to prevent errors and unwanted events it causes can improve patient safety. On the other hand, instead of developing a culture of blame and punishment for errors, recognizing vulnerabilities and creating viable solutions should be the primary goal (Buzink SN et al, 2010).

Measuring medical errors scientifically is quite difficult in many ways. Confusion in the terminology of medical errors, difficulties in the registration system, legal reservations prevents the registration of medical errors (E.G.G. Verdaasdonk et al, 2007). On the other hand, health professionals also experience profound psychological effects such as anger guilt inability depression, and suicide due to events perceived as real mistakes or mistakes (Rodziewicz Thomas L et al, 2021). Fear of punishment deters health workers from reporting their mistakes. However, unreported errors contribute to the sleep of potential events that will harm patient health. A culture of blame and punishment can cause a medical staff to hesitate to report the problem, minimize the problem by their means, and even avoid documenting

the problem (Buzink SN et al, 2010). All these actions can lead to a thriving and growing cycle of medical errors. When this chain of errors occurs, it can damage the reputation of the health institution and employees. Some experts have noted that the word medical error is a word that feeds an overly negative hostile and blame culture (Reason J, 1990)). Even the use of the word error can be the cause of psychological destruction, which will reduce the motivation of health workers or even lead to the point of quitting the profession. It would be wise to limit the use of such a negative term. Eliminating negative connotations, on the other hand, can have negative consequences for patients. It is also an undeniable fact that there is a public and legal intolerance to medical errors. The fact that some of these mistakes cannot be prevented is decidedly ignored in public. Legal and medical institutions must work together to eradicate the culture of crime while maintaining accountability. Once these challenges are overcome, health institutions will not be constrained to measure their goals to improve patients, even in adverse outcomes.

ERROR DEFINITIONS

” Error, omission, undesirable event " are frequently used words for medical error. In the education of health workers, these words should be taught by being clearly defined as a term. Medical error data can only be recorded correctly in this way. Reporting error-prone situations will reduce future errors. Some important current error definitions are as follows (Rodziewicz T, 2021):

Active error: these are errors that occur between the patient and a health worker at the point of contact and one aspect of the system. It is done by people on the front lines, such as clinicians and nurses. Surgery on the wrong side of the kidney is an example of active error.

Undesirable event: a condition caused by prolonged hospitalization or the nature of the health system. It is the occurrence of an undesirable condition in medical and surgical treatment rather than the patient's medical condition. The preventable ones of these events can be attributed to medical errors. But the development of subglottic stenosis in a patient who remains intubated for a long time should be considered as an undesirable event, not a medical error.

Hidden errors: these are errors in the system or process design. It can be faulty installation, improper equipment maintenance, ineffective organization. Hidden errors are sleeping accidents. An active human error can reveal a sleeping accident. These errors are present, but they may not be noticed for a long time, and the bad effect may not be felt.

Medical error: can be defined as negligence that contributes to or may contribute to an unintended outcome when planning and performing a procedure.

Negligence: the inability of a healthcare worker caring for a patient to provide a reasonable standard of care. For example, the doctor may not have looked at the patient's results. An undesirable event due to negligence can be exemplified by an injury caused by

substandard medical management. There may be a definition for the legal dimension.

Potential error: can be defined as situations that may cause a negative presence in the patient, but have not yet caused problems. It can also be interpreted as a near mistake..it provides fursuits for developing preventive strategies and actions. so it needs to be studied on the same plane as negative events.

Mistakes that should never happen: the classic example of this event is the development of wrong-zone surgery. It is serious and must be reported. It is the events that cause the most legal problems. The patient may die or become crippled, and the physician may be barred from the profession. It may be related to patient care management. It may be related to the device or medical product, the environment, patient protective factors, surgery, and radiology.

Errors that seem useful but cause harm: asking for a long-term examination from a patient who has lost blood can be an example of these errors. Your patient. It could cause his death.

OPERATING ROOM ERRORS

Reason has studied major accidents, such as the nuclear accident on a Pacific island in 1979. After all, he found that before major accidents and unwanted events, there was a chain of small events that seemed seemingly insignificant. That is, it begins with examining events that seem trivial to prevent future major accidents and create a defense system (Reason J, 1990). According to the scheme, which

Reason describes as the 'Swiss cheese model', errors in an organizational system cause undesirable events by exploiting vulnerabilities in security barriers. G.G. Verdaasdonk et al, 2007).

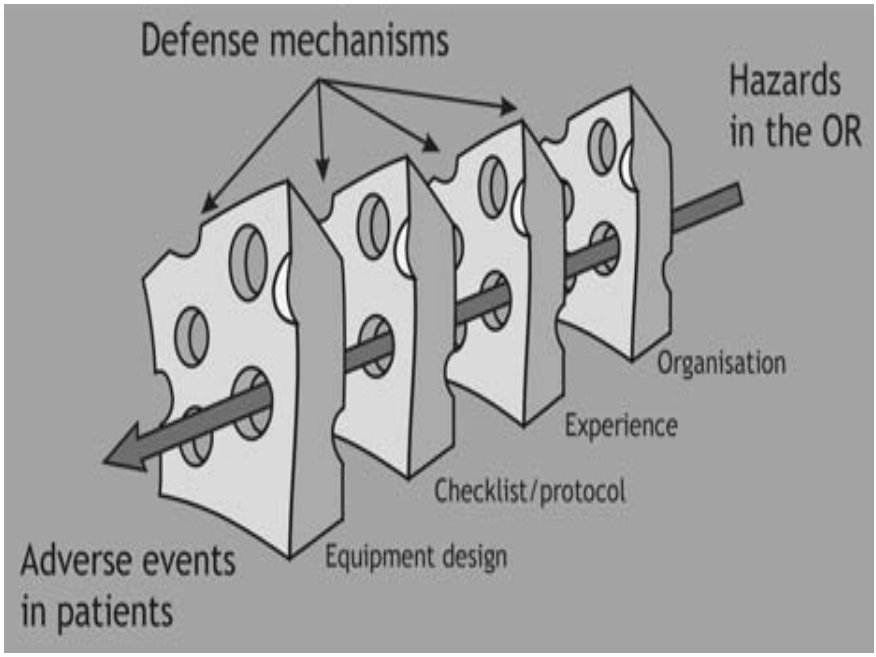

Figure 1. Swiss cheese model (arrangement for medical error), cheese slices hospital organization (team) experience, checklists/protocols, and equipment design, such as defense mechanisms represents. Holes, on the other hand, represent undesirable events, and these are weaknesses in defenses. Events in a complex environment (such as operating rooms) can cause an accident trajectory (big Arrow) and lead to negative events.

Since its identification, the Swiss cheese model has provided outstanding benefits in managing deconstruction processes in explaining the relationship between errors and unintended events. In particular, it has been very useful in preventing unwanted events in the aerospace sector and nuclear facilities with complex organizational schemes, where errors affect the entire organizational chart and lead to

irreversible problems (E.G.G. Verdaasdonk et al, 2007). The health care system also contains a complex organizational chart, in which very complex processes work. Numerous studies have been conducted for processes starting from a patient's admission to the hospital. Some of these studies give us information about possible errors. 44,000 people die each year in the United States (USA) from medical errors (Rodziewicz Thomas L.) et al, 2021). It has been reported that this figure may even be as much as 98,000 (Mascioli S, 2016). These figures are even higher in annual motor vehicle accidents in this country. The figures are quite dramatic. Therefore, safety barriers proposed by the Universal accident model for errors and undesirable events in different sectors have been developed and useful models have been designed in medical processes (Figure 1) (E.G.G. Verdaasdonk et al, 2007).

Medical errors and undesirable events mostly occur in operating rooms (6Kuzdan BC et al, 2019). In some studies, errors are classified as negligence and operation errors in 2 ways (Rodziewicz Thomas L. et al, 2021). As an example of negligence errors, a patient's fall during a transplant as a result of not being placed on a stretcher correctly and not being fixed can be defined as an undesirable event as a result of negligence. As a result of the incorrect insertion of cautery plaque into a patient, the formation of burns in the patient can be defined as a surgical error. Rather, we will address technical errors that occur in the operating room, where undesirable events occur most often, as well as undesirable events caused by them, and suggestions for solutions.

Every year, more than 200 million surgical procedures are performed all over the world. Surgical errors are responsible for a significant number of undesirable events. At least 4,000 surgical errors occur in the United States each year (Kuzdan BC et al, 2019). The interesting thing about surgical errors is that these errors are more common before and after surgery than mistakes made in the operating room (James JT, 2013). Some of the causes of surgical errors listed in the literature are as follows: the lack of adequate surgical training, the lack of standardized rules and regulations, the surgeon – anesthesiologist-the lack of sufficient communication between the staff helpful, the lack of safe systems and protocols, is not in a hurry to quickly complete cases (Bulbuloglu, S, 2017b). Verdaasdonk et al. recorded the procedures with a video recording system that they developed. They identified the errors by tracking them to an expert commission. Accordingly, 84% of unintended incidents were due only to equipment-related problems. In half of these problems, the equipment was working poorly, and in 20%, the cause of the problem was unclear. The conclusion is that despite all efforts, we are faced with numerous errors, the cause of which cannot be determined. The shortcomings that need to be explored in this regard are like a galaxy waiting to be discovered.

Technical errors in operating rooms are generally examined in 3 parts (E.G.G. Verdaasdonk et al, 2007). These are classified as device problems, instrument problems, and human errors:

Device problems: devices used during laparoscopy, a complex procedure, require installation information. Since the installation of the device requires experience, errors and problems that are often encountered in complex procedures are related to the devices (4). A monitor, camera head, and light source are used as devices in the laparoscopy process. The most common device problem is due to the monitor (Rodziewicz Thomas L. et al, 2021). Given that there are about 5,000 types of medical devices used by millions of healthcare providers worldwide, errors with the device are inevitable.

Instrument problems: instrument errors have been reported in the literature at different rates. In a study in the literature, the authors reported that 37% of their surgeries had an instrument problem, and this was all about the quality of the material used. Another study reported this rate as 20% (Denver, C.O. (2015).).

Human errors: human errors are another problem in endoscopic surgery. Verdaasdonk et al found 31 position errors, 6 installation errors, and 18 connection errors during 30 laparoscopic surgery procedures. It has also been reported in the literature that position errors are the most common errors (1 Rodziewicz Thomas L. et al, 2021). The positioning of equipment is often related to monitors. Proper placement of screens requires extensive planning. Regardless of the mounting system, monitors must usually be placed before the surgeon can begin work. Surgeons share the view that two monitors are essential both for ergonomic reasons and to ensure optimal vision. However, for logistical reasons, a second monitor is not

always available, and the repositioning of monitors that are present after the surgeon initiates the procedure is also recorded as a procedural event. But it takes time to fix, and it disrupts concentration (E.G.G. Verdaasdonk et al, 2007).

Human and organizational errors related to pathology examples

Pathology sample safety in the operating room two issues are of great importance in the safe management of pathology samples in the operating room.

1) samples should not change until they arrive at the laboratory. In such a case, for example, the possibility of pre-removal and may lead to new surgery for the patient.

2) an example of an altered pathology increases the risk of the doctor's error. As a result of an incorrect diagnosis, incorrect treatment can be applied, and its return to the patient can be very serious damage. Safe surgical pathology Material Management in the operating room requires attention, collaboration, team coordination, skills, and knowledge, for example, from receipt to transport to the laboratory (James JT, 2013). For this purpose, the material removed by the surgeon must be fastened, labeled, recorded, and sent to the laboratory in accordance with its technique, in the material container and solution, without deteriorating integrity (Bülbüloğlu S, 2017A). A meta-analysis study on this topic found that in more than 1,850 materials, 0.05% of errors occurred due to incorrect or incomplete patient information being entered during sample labeling, and 7.7%

due to the absence of a request form or result report (Bülbuloğlu S, 2017B).

The most common mistakes are inaccuracies in patient information:

1) labeling errors or untagged samples,

2) inappropriate prompt form,

3) it has been stated that unsafe pathology due to unsafe sample sending is sample sending (Steelman, V.M, 2016).

Maintenance errors during surgery

Anesthetics can lead to hypothermia. Body temperature should be constantly displayed, and temperature changes should be noticed early (Bashaw m.A., 2016). Protective measures should be taken when using Scopia and radiation measurements should be made. Electrosurgery should be performed safely with the participation of trained and equipped nurses. Vital assessments and records should be kept continuously throughout the operation. Changes should be implemented in the specified health care (Bülbüloğlu, P, 2017a). A sterile area should be installed by specially trained personnel throughout the operation. If the sterile chain is broken, it must be intervened. The surgical team must apply bone, Apron, gloves, and other aseptic techniques. Asepsis should be provided with the appropriate solution when cleaning the skin of the area where the surgical incision will be applied. If there is an existing wound, it should be closed using a wound cover. The surgical team should pay attention to covering the surgical veil in accordance with aseptic

techniques (Steelman, V.M, 2016). Unplanned changes in operations, emergencies, overweight patients, miscalculated gauze and tools, multiple surgeries, communication disruption are considered difficult processes that compromise the safety of the operating room and management (Bülbüloğlu, P, 2017a).

CURRENT SOLUTION APPROACHES

During emergency surgery, technical problems can be difficult to solve. For this reason, some approaches before surgery can prevent problems. All technical equipment should be reviewed, if necessary, confirmed that there is no technical problem before the operation, personnel training, instrument and device checklist, and protocol should be established within this framework. Especially the checklist can provide an effective solution in preventing technical problems E.G.G. Verdaasdonk et al, 2007).

In emergency surgery, information interviews scheduled in his room with the patient and his relatives, which are part of preoperative health care, cannot be held. Time must be devoted to this. The surgical team should take responsibility for preventing any situation that endangers patient safety, knowing that the patient is vulnerable throughout the procedure (Steelman, V.M, 2016). Body regions at risk for injury during surgery in the prevention or reduction of surgical emphasis in writing and verbally, the entire surgical team and the patient's attention all team members have been reported to provide protection from pressure ulcers and other risks (Denver C.O. , 2015). Bone protrusions in various parts of the body should be

supported by soft or air pillows for pressure ulcers. The patient taken to the operating table should be connected with a safety belt that will be fixed to the table to prevent falls. The arches should be fixed so as not to cause tissue ischemia or nerve damage, and supported with soft support materials to prevent trauma to the bone protrusions (Denver C.O., 2014). Surgical nurses should keep records fully during the operation, covering all health care processes (improper position, prevention of pressure ulcers, use of heat probe, presence of implants, drains and wound cover, how many times a tourniquet is used, surgical plaque position, etc.) should report (Bülbüloğlu S, 2017b). In addition to being a basic dimension for the surgical team, recording every application in Octoberncreases the prominence of health care by creating data for managers, policymakers, and finance managers (Bülbüloğlu, P, 2017a). The process of moving the sample safe surgical pathology; for example, are to be received, where the boundaries of the detection, removal, nomenclature, identifying and using an appropriate protective solution in the sample container, labeling, maintaining the integrity of the sample to the pathology lab in the process of moving and keeping complete records includes (N Akansel, 2015). At the stage of removing surgical pathology samples, labeling and sorting together with pathology documents, the necessary measures should be taken and these processes should be carried out in accordance with quality standards (Bülbuloğlu S, 2017b).

Sonia Buzink et al. used an equipment checklist they called‘ pro-check". However, the team reported that they had established a successful ‘safety culture.’ Verdaasdonk et al. they reported a 53% reduction in technical problems thanks to the checklist they developed. As a result of the study, they proposed several approaches to prevent problems, which are:

(1) redesign of equipment,

(2) improvement of training/qualification controls,

(3) Use of protocols and checklists.

Redesigning equipment and systems is an expensive and slow process. However, there are systems integrated into the new operating room design that can provide solutions to some problems in existing equipment and systems. Unfortunately, many hospitals do not have sufficient financial resources for the operating theatres of the future. In addition, surgical procedures that require an increased system of advanced technologies often create unpredictable new problems. For example, robotic surgical equipment that begins to replace laparoscopy is very expensive, the training and installation processes are long and complex. The new problems it will bring are also not yet sufficiently known.

Proper training to prevent unwanted incidents in operating rooms has great potential. The advantage of implementing a training program is that it provides the opportunity to deconstruct a standardized safety culture among staff (James JT, 2013). It should be noted that training

can be time-consuming and knowledge and skills are lost over time. Continuity in education is therefore critical.

The third approach, the use of checklists or protocols, can provide a quick and inexpensive solution to prevent minor incidents. A relatively short training focused on the use of equipment checklists is expected to help reduce the number of incidents.

It is important to note that decongestant for pre-operative safety measures (Mascioli S, 2016) may be the most important surgical safety measure, and a preoperative pause involving all members of the surgical team is useful. Having time-outs while the patient is on the operating table before the operation begins will minimize costly errors in the right place, the right procedure, and the right patient.

As a result, examining and evaluating the Health Organization system as a whole is the only way to combat the numerous errors that have been detected or hidden.

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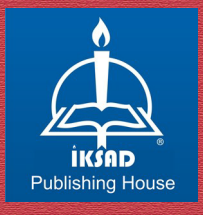

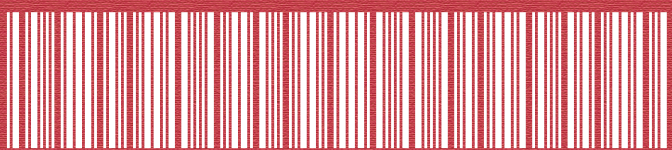

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