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Chapter 1

IN SILICO STUDIES AND STRUCTURE ACTIVITY RELATIONSHIPS OF PICOLINIC ACID DERIVATIVES AS A-GLUCOSIDASE INHIBITORS

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INTRODUCTION

Diabetes is a chronic metabolic and non-communicable disease, one of the five leading causes of death in the world with multiple etiologies (Wild et al., 2004:1047). Diabetes mellitus (DM) characterized by a high level of blood glucose, is known as a common metabolic degenerative disease (Alam, 2012:398). This disease is a clinical syndrome of social importance characterized by hyperglycemia due to absolute or relative insulin deficiency (Aguwa, 2004:125; Balan et al., 2017:732). Although the type 2 diabetes (T2DM) class is complex in nature, it is stated that insulin resistance, genetic and environmental factors are associated with this type of diabetes due to impaired insulin secretion (Iwahashi et al., 1998:45; Kumari and Cain, 2012:70). Many strategies adopted to treat diabetes include α-glucosidase inhibition in the gastrointestinal tract associated with slowing carbohydrate digestion in the gut and delaying glucose absorption (Tripath et al., 2014:13). Many studies on diabetes show that α-glucosidase inhibitors delay the progression of T2DM (Rhabasa-Lhoret and Chiasson, 2004).

Heterocyclic compounds containing nitrogen atoms, such as picolinic acid, are one of the important classes of organic compounds due to their structural similarities with most protein-containing enzyme structures in our body, and these compounds are known to be biologically important and used for the design of medicinal compounds (Khan et al., 2008:40; Iqbal et al., 2009:31). Molecules containing these groups are known to have many pharmacological and biological properties such as anti-inflammatory, antibacterial, antipyretic, antifungal, anticancer, and antimalarial activities (Xia et al., 2015:83; Ghosh et al., 2018:252; Sönmez et al., 2019:829). Synthesis and physico-chemical properties of picolinic acid derivative, especially such as insulinomimetic activity, it attracts attention in many application areas.

It is well-known that in silico molecular docking studies lead to the screening, identification and development of more active bioactive molecules against many known diseases. In the present chapter, picolinic acid and some 6-substituted (-fluoro, -chloro, -bromo, -methyl, -nitro) derivatives were selected as potential α-glucosidase inhibitors and their interactions with α-glucosidase enzyme and structure activity relationship were investigated by the AutoDock4 program.

METHODS

The target α-glucosidase enzyme (S. cerevisiae isomaltase (PDBID: 3A4A)) was chosen rigidly and molecular docking was performed with

the AutoDock4 program implemented via the graphical user interface AutoDockTools (ADT1.4.6) (Morris et al., 1998:1639).

All calculations for substrate-target enzyme insertion were performed using the Lamarckian genetic algorithm method (Solis and Wets, 1981:19). 2D and 3D structures showing the interactions between enzyme active site and substrate were drawn in Discovery Studio 4.0 (Systèmes, 2016).

DISCUSSIONS

The protein-ligand interactions, distance values and calculated inhibition constants (Ki) for picolinic acid and its derivatives are given in Table 1. According to docking results, selected picolinic acid and its derivatives showed α-glucosidase inhibitory activity. The calculated inhibition constants (Ki) values were between 1.11 mM and 2.68 mM. Among them, 6-nitropicolinic acid exhibited the strongest inhibitory activity against α-glucosidase with the Ki value of 1.11 mM, while 6-fluoropicolinic acid showed the weakest α-glucosidase inhibition with the Ki value of 2.68 mM.

Tablo 1: *Protein-ligand interactions, distance values and calculated inhibition constants (Ki) for picolinic acid and its derivatives*

Substrate	Receptor	Interaction	Distance (Å)	Ki(mM)
PicH				*2.59*
-C=O	*GLY-564*	*Convent. H-Bond*	*3.29*	
-COO-	*LYS-568*	*Convent. H-Bond*	*2.65*	
-COO-	*PHE-364*	*Carbon H-Bond*	*3.19*	
Pyr	*LYS-568*	*Pi-Alkyl*	*4.65*	
6-F-picH				**2.68**
-COO-	*LYS-45*	*Convent. H-Bond*	*2.71*	
Pyr	*GLU-407*	*Amide Pi-Stacked*	*4.63*	
Subs.	*TYR-48*	*Van der Waals*		
6-Cl-picH				**1.65**
-COO-	*LYS-466*	*Convent. H-Bond*	*2.94*	
-C=O	*PRO-467*	*Carbon H-Bond*	*2.82*	
Pyr	*LEU-471*	*Pi-Donor H-Bond*	*3.91*	
Pyr	*LEU-471*	*Pi-Alkyl*	*4.81*	
-Cl	*TYR-470*	*Pi-Alkyl*	*4.94*	

Substrate	Receptor	Interaction	Distance (Å)	Ki(mM)
6-Br-picH				**1.61**
-C=O / -COO-	*LYS-45*	*Convent. H-Bond*	*3.05 / 2.95*	
Pyr	*GLU-47*	*Amide Pi-Stacked*	*4.13*	
-Br	*ILE-485*	*Alkyl*	*4.58*	
-Br	*TYR-48*	*Pi-Alkyl*	*4.78*	
6-Me-picH				**1.62**
-C=O	*LYS-466*	*Convent. H-Bond*	*2.67*	
-C=O	*PRO-467*	*Carbon H-Bond*	*2.93*	
Pyr	*LEU-471*	*Pi-Donor H-Bond*	*3.47*	
Pyr	*LEU-471*	*Pi-Alkyl*	*4.64*	
-CH3	*TYR-471*	*Pi-Alkyl*	*4.95*	
6-NO2-picH				**1.11**
N=O (-NO2)	*LYS-406*	*Convent. H-Bond*	*2.66 / 2.69*	
-C=O	*PRO-467*	*Carbon H-Bond*	*3.24*	
Pyr	*LEU-471*	*Pi-Alkyl*	*5.11*	

It can be observed the structure–activity relationship (SAR) from Table 1: (i) all 6-substituted picolinic acid derivatives (except 6-fluoro) showed stronger α-glucosidase inhibitory activity than picolinic acid; (ii) the electron-withdrawing group (nitro) increased the inhibitory activity more than electron-donating groups (methyl); (iii) the halogen series at the 6- position exhibited relationship between increasing polarizability/ size and high inhibitory activity (for polarizability/size: Br > Cl > F, for inhibition: Ki values of 6-Br, 6-Cl and 6-F-picH were calculated as 1.61 mM, 1.65 mM and 2.68 mM, respectively).

According to the docking results of the selected molecules (Table 1), various interactions such as H-bonds, different pi-stacking and van der Waals were observed between the pyridine rings, carboxyl groups, and 6-position substituents and amino acid residues of the glucosidase enzyme, as expected.

2D and 3D structures showing the interactions between enzyme active site and picolinic acid, 6-nitropicolinic acid, and 6-methylpicolinic acid are given Figure 1-3, respectively.

Regarding the docking results of picolinic acid, conventional H-bond between carbonyl group and GLY-564(3.29 Å)/LYS-568(2.65 Å), carbon H-bond between carbonyl group and PHE-364(3.19 Å), and pi-alkyl interactions between pyridine ring and LYS-568(4.65 Å) were detected (Fig.

1).

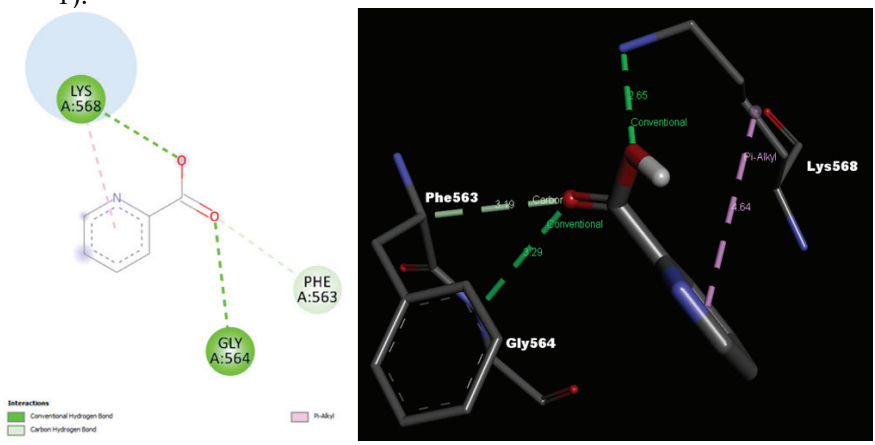

Figure 1: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and Picolinic Acid*

According to the docking results of 6-nitropicolinic acid, conventional H-bond between nitro group and LYS-406(2.66 and 2.69 Å), carbon H-bond between carbonyl group and PRO-467(3.24 Å), and pi-alkyl interactions between pyridine ring and LEU-471(5.11 Å) were observed (Fig.

2).

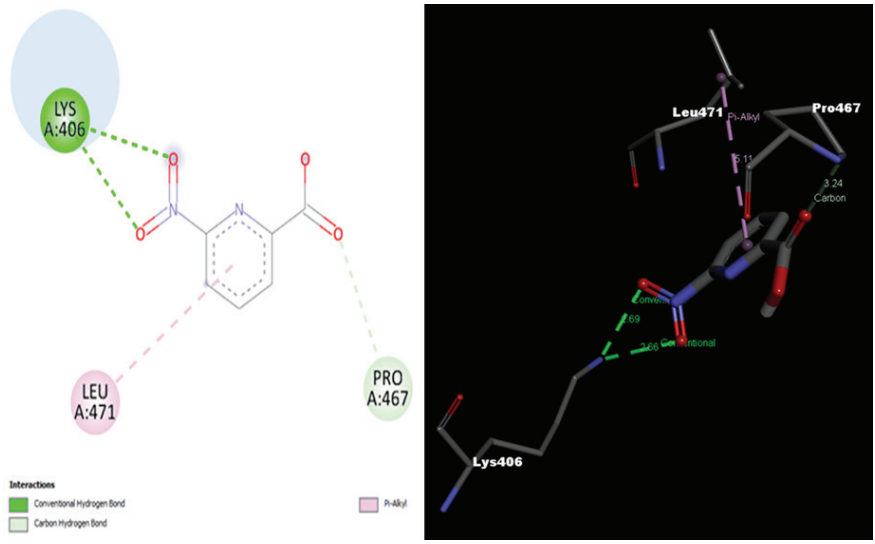

Figure 2: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and 6-Nitropicolinic Acid*

Regarding to the docking results of 6-methylpicolinic acid, conventional H-bond between carbonyl group and LYS-466(2.67 Å), carbon H-bond between carbonyl group and PRO-467(2.93 Å), pi-alkyl interactions between pyridine ring and LEU-471(4.64 Å) and pi-alkyl interactions between methyl group and TYR-471(4.95 Å) were observed. Moreover, different from others, pi-donor H-bond interactions between pyridine ring and LEU-471(3.47 Å) were detected (Fig. 3).

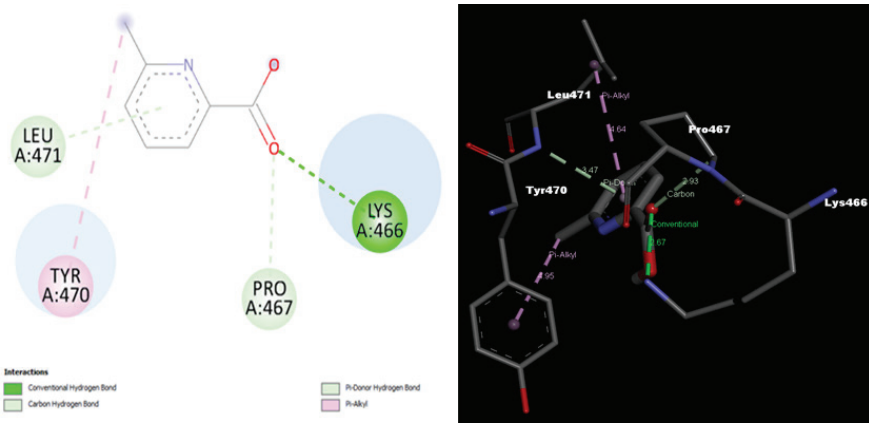

Figure 3: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and 6-Methylpicolinic Acid*

CONCLUSION

In conclusion, this chapter has demonstrated that the picolinic acid and its 6-substituted derivatives exhibited α-glucosidase inhibitory activity and they can be selected for synthesis of various α-glucosidase inhibitors as starting material. Especially, it can be considered that some halogens such as -Cl and -Br and electron-withdrawing group such as -NO2 can be preferred due to their various interactions with amino acid residues in enzyme active site. Furthermore, the binding of electron-donating or electron-withdrawing substituents to heterocyclic compounds is important as they directly affect the inhibitory properties.

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Chapter 2

piRNAs AND THEIR ROLES IN HUMAN CANCERS

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Introduction

The RNA world and the functions of different transcripts have been understood in more detail together with the rapid development observed in technology used in molecular biology and genetics. Information beyond the regulation of gene expression by transcription factors and other proteins have been revealed by extensive genome studies such as Encyclopedia of DNA Elements (ENCODE). These informations are an understanding that a very large part of the genome is non-coding having important roles. Transcription, chromatin structure and histone modification regions have systematically been mapped with the ENCODE Project. These data provided an understanding of the functions of 80% of the genome, except for the protein-coding regions. Most of the candidate regulatory elements discovered are physically related to each other and to the expressing genes. In addition, this information also provide information about gene regulation mechanisms (ENCODE Project Consortium, 2012).

Although 90% of the human genome is transcribed, only 2% of the genome sequence is known to be protein-coding genes (Gibb et al., 2011). It has been seen that the transcriptome contains various functional non-coding RNA classes that have an important role in human diseases and function as regulators of gene expression, together with developing molecular techniques (Taft et al., 2010). Non-coding RNAs are divided into two main classes including long non-coding RNAs and small non-coding RNAs. These RNAs act as regulators of gene expression (Lewis et al., 2003). Long non-coding RNAs are a class of RNA that contains more than 200 nucleotides (Derrien et al., 2012). PIWI (P-element-induced wimpy testis)-interacting RNAs (piRNAs), microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs), short interfering RNAs (siRNAs) and ribosomal RNAs (rRNAs) are of small ncRNAs class (Chan and Tan 2018).

Despite advances in chemotherapy, radiotherapy and surgical techniques, the search for more effective treatments for cancer therapy continues. In recent years, piRNAs have been found to be important in the formation and progression of cancer. Therefore, piRNAs are seems to be promising candidates in cancer therapy. Investigation of the molecular roles of piRNAs in the diagnosis, prognosis and treatment of cancer has gained great importance.

Discovery of piRNAs

The first piRNAs were identified in Drosophila testes in 2001 and were named Repeat Associated RNAs (rasiRNAs). However, it was renamed as piRNA after understanding the interactions between PIWI and

them (Aravin et al., 2001). First mammalian piRNAs, which has 25-32 nt long, have been discovered in rat and mouse spermatogenic cells by different research groups in 2006 (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006). The presence of piRNAs in humans was first detected in ovary and testis tissues (Williams et al., 2015). As illustrated in Figure 1., the source of piRNA transcripts is various for instance protein-coding regions, 3′- and 5′-untranslated regions (UTR), small nucleolar RNAs and long non-coding RNAs besides transposable elements (He et al., 2015; Keam et al., 2015; Martinez et al., 2015). In the transcriptional level, piRNAs can directly change chromatin structure via DNA methyltransferases (DNMTs) by interacting with PIWI proteins. It can cause silencing of transposable elements and target genes via DNMTs and PIWI proteins (Wu et al., 2020). In the post-transcriptional level, piRNAs and PIWI can cause mRNA degradation (Rouget et al., 2010).

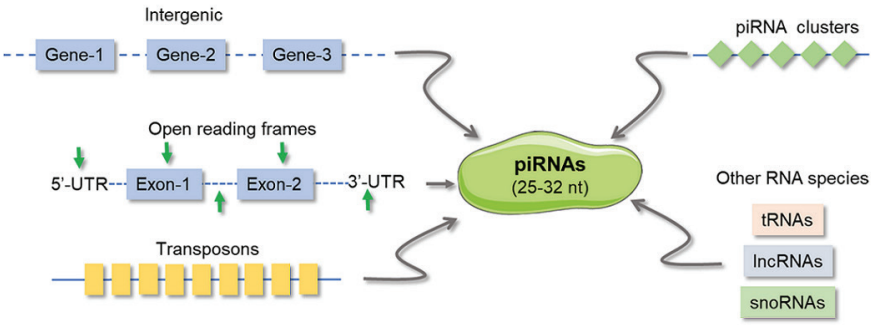

Figure 1. *Genomic sources of piRNAs (Adapted from Krishnan &Damaraju 2019).*

Biogenesis of piRNAs in human

A web resource on piRNAs has been showed that there are 23439 piRNA genes in the human genome (http://pirnabank.ibab.ac.in/pirnabank.html). piRNA biogenesis occurs in two stages: (i) piRNA transcription in nucleus and post-transcriptional modifications in cytoplasm and (ii) processing of primary piRNA in ping-pong cycle. Briefly, RNA polymerase II transcribes piRNAs cluster. The precursor chains of piRNAs passing into the cytoplasm are loaded onto the PIWIL1 protein. The piRNA is then loaded onto the PIWIL2, which is bound by tudor domain-containing protein 1 (TDRD1). After the processing of the 5′ and 3′ ends of the primary piRNAs, the piRNAs enter the ping-pong cycle where the second processing will occur. The resulting piRNA-PIWIL2 complex binds to another piRNA strand. The secondary piRNA loaded on the PIWIL2 protein forms a complex with PIWIL4. This complex binds to the target mRNA

or transposons and regenerates primary piRNA (Figure 2; Balmeh et al., 2021). piRNA mediated silencing occurs via either histone modification and DNA methylation at the transcriptional or mRNA degradation at the posttranscriptional levels (Figure 3; Jia et al., 2022).

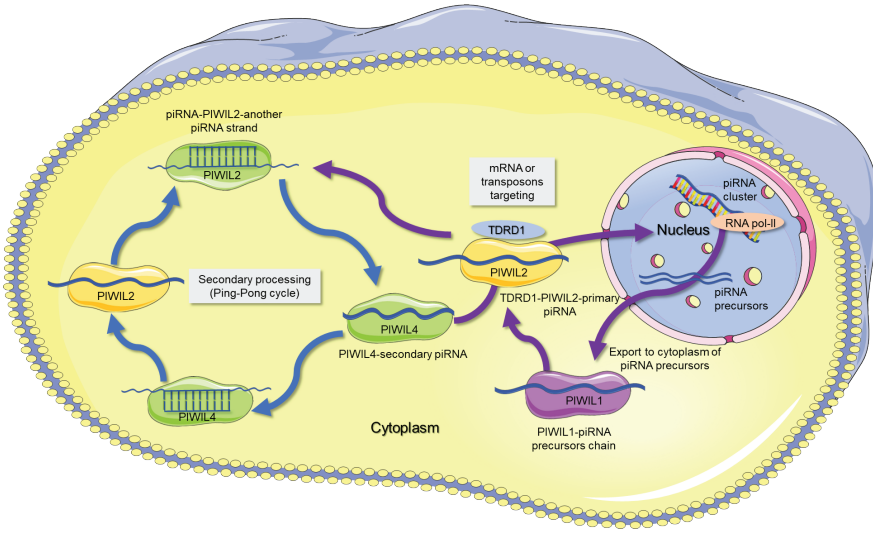

Figure 2. *piRNAs biogenesis in human (Adapted from Balmeh et al., 2021).*

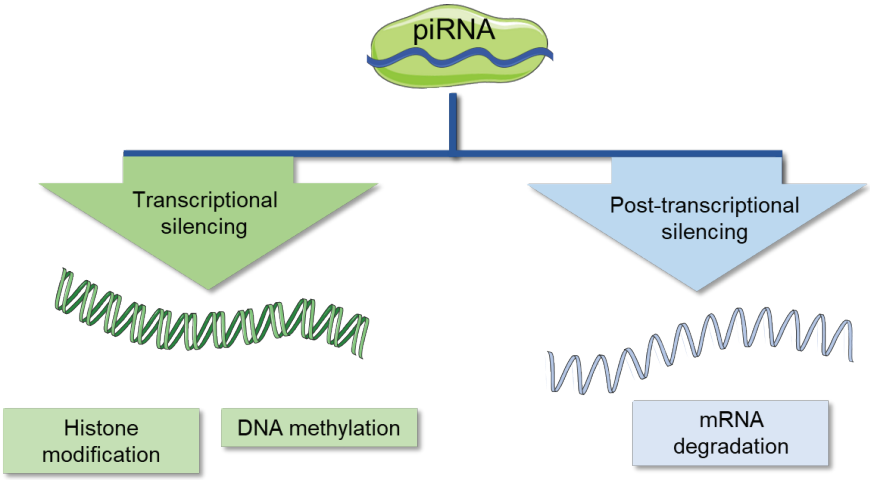

Figure 3. *Roles of piRNAs at transcriptional and post-transcriptional level.*

Abnormal expression of piRNAs in cancer

There is evidence that piRNAs, which are known to be genome protectors in germ cells, are expressed in various somatic cells (Tosar et al., 2018). Although piRNAs were initially thought to be important in germ cells, it was later seen that the expression of piRNAs changed in different types of cancer. Studies have shown that some piRNA expression changes in cancer are associated with clinical data and prognosis of the disease (Tan et al., 2019; Iyer et al., 2020). Previous literature indicated that piRNAs and PIWI proteins play critical roles in cancer. Therefore, piRNAs may be important for the diagnosis, prognosis and treatment of cancer. Advances in sequencing techniques lead to a better understanding of the non-coding part of the genome. This has led to the development of cancer treatment options, especially for other diseases.

piRNAs are important in DNA methylation not only at transposons but also at non-transposon sites. In recent years, epigenome-wide analyzes have been performed to identify genes methylated by piRNAs (Fu et al., 2014). It is known that piRNAs may have oncogenic and/or tumor suppressor roles (Cheng et al., 2012; Zhang et al., 2020). The short sequences of piRNAs allow them to pass through the cell membrane without any degradation. This allows piRNAs to be detected in various samples such as serum, plasma, urine and sputum of patients (Yang et al., 2015). Therefore, piRNAs can be easily detected in these samples for diagnosis and prognosis of the disease.

Numerous studies have been conducted on the roles of other small non-coding RNAs in the pathogenesis of cancer. However, there are limited studies on piRNAs and cancer. Studies on cancer and piRNA can provide information about the formation mechanisms and development of cancers and may lead to the emergence of new therapeutic approaches. There is need for more approaches that are effective in the diagnosis and treatment of cancer. piRNAs with abnormal expression in various cancers are summarized in Table 1.

Table 1. *piRNAs having abnormal expression levels in various cancers*

Cancer type	***piRNA***	***Expression***	***Reference***
Lung cancer	*piR-651*	*Upregulated*	*Li et al., 2016*
	piR-34871	*Upregulated*	*Reeves et al., 2017*
	piR-52200	*Upregulated*	
	piR-35127	*Downregulated*	
	piR-46545	*Downregulated*	
	piR-55490	*Downregulated*	*Peng et al., 2016*

Cancer type	piRNA	Expression	Reference
Lung cancer	*piR-211106*	*Downregulated*	*Liu et al., 2021*
	piR-26925 *piR-5444* *piR-30636* *piR-8757*	*Upregulated*	*Li et al., 2021*
Prostate cancer	*piR-002468* *piR-158533* *piR-349843* *piR-382289*	*Upregulated*	*Peng et al., 2021*
	piR-017184 *piR-001773*	*Upregulated*	*Zhang et al., 2020*
	piR-19166	*Downregulated*	*Qi et al., 2020*
Breast cancer	*piR-36712*	*Downregulated*	*Tan et al., 2019*
	piR-932	*Upregulated*	*Zhang et al., 2013*
	piR-20582 *piR-20485* *piR-4987* *piR-20365*	*Upregulated*	*Huang et al., 2013*
Colon cancer	*piR-020365* *piR-017724* *piR-017723* *piR-004153* *piR-001311*	*Downregulated*	*Qu et al., 2019*
	piR-54265	*Upregulated*	*Mai et al., 2018*
	piR-5937 *piR-28876*	*Downregulated*	*Vychytilova-Faltejskova et al., 2018*
	piR-24000	*Upregulated*	*Iyer et al., 2020*
	piR-651	*Upregulated*	*Cheng et al., 2011*
Pancreatic cancer	*piR-017061*	*Downregulated*	*Xie et al., 2021*
Hepatocellular cancer	*piR-Hep1*	*Upregulated*	*Law et al., 2013*
	piR-00823 *piR-LLi-30552* *piR-013306* *piR-020498*	*Upregulated*	*Rizzo et al., 2016*

Cancer type	piRNA	Expression	Reference
Ovarian cancer	*piR-52207*	*Upregulated*	*Singh et al., 2018*
Bladder cancer	*piR-60152*	*Downregulated*	*Chu et al., 2015*
Multiple myeloma	*piR-823*	*Upregulated*	*Yan et al., 2015*
	piR-004800	*Upregulated*	*Ma et al., 2020*
Glioblastoma	*piR-8041*	*Downregulated*	*Jacobs et al., 2018*
	piR-12488 *piR-12487* *piR-9491* *piR-1849*	*Downregulated*	*Bartos et al., 2021*
Gastric cancer	*piR-1245*	*Upregulated*	*Zhou et al., 2020*
	piR-651	*Downregulated*	*Cui et al., 2011*
	piR-823	*Downregulated*	*Cui et al., 2011;* *Cheng et al., 2012*
	piR-651	*Upregulated*	*Cheng et al., 2011*
Kidney (renal) cancer	*piR-823*	*Downregulated*	*Iliev et al., 2016*
	piR-51810 *piR-34536*	*Downregulated*	*Zhao et al., 2019*
	piR-32051 *piR-39894* *piR-43607*	*Upregulated*	*Li et al., 2015*
Cervical cancer	*piR-14633*	*Upregulated*	*Xie et al., 2022*

Lung cancer

Lung cancer is the leading cause of cancer-related deaths. While the overall five-year survival rate is 22%, the five-year survival rate for localized tumor is 60%. However, only 24% of patients are diagnosed at an early stage (American Cancer Society, 2022). As in all cancers, miRNAs have been studied extensively in lung cancer; but, there are very limited studies on piRNAs. piR-651 is one of the highly expressed piRNAs in the lung cancer cell line. In these cells, piR-651 has increased proliferation by increasing cyclin D1 and CDK4 expressions (Li et al., 2016). Microaarray studies of lung cancer cells overexpressing RASSF1C, important in the pathogenesis of various cancers, showed more than 500 aberrantly expressed piRNAs. Moreover, expressions of upregulated piR-34871 and piR-52200, and downregulated piR-35127 and piR-46545 in lung tumor correlated with results of microarray analysis (Reeves et al., 2017). Ex-

pression of piR-55490 has been significantly downregulated in lung cancer tissue compared with surrounding normal lung specimens and lung cancer cells compared with normal lung cells. Also, it has been found that piR-55490 caused suppression of the Akt/mTOR pathway by binding to the 3′-UTR of mTOR mRNA in lung cancer cells. The survival rate is higher in the group with high piR-55490 expression compared to the group with low piR-55490 expression (Peng et al., 2016). Knockdown of piR-651 suppressed cell proliferation, migration, invasion and induced apoptosis in A549 and HCC827 non-small cell lung cancer (NSCLC) cells (Zhang et al., 2018). Expression level of piR-211106 is decreased in lung adenocarcinoma compared to non-cancerous tissues. It has been shown that piR-211106 suppressed proliferation, migration and induced apoptosis in lung cancer. In addition, this piRNA showed anticancer effect synergistically with cisplatin in lung cancer. RNA immunoprecipitation and RNA pull down assays have shown that piR-211106 could inhibit pyruvate carboxylase mRNA and protein expression. Therefore, piR-211106 suppress lung cancer progression and increases chemotherapy sensitivity by inhibiting pyruvate carboxylase. This suggests that piR-211106 may be a diagnostic and therapeutic target in lung cancer (Liu et al., 2021). Expressions of piR-26925, piR-5444, piR-30636 and piR-8757 were increased in lung adenocarcinoma tissues compared with surrounding non-tumor tissues. It has also been shown that piR-26925 and piR-5444 levels are higher in serum exosome samples of lung adenocarcinoma patients when compared to healthy control serum exosome samples (Li et al., 2021). The different expression of these piRNAs in serum samples shows that they can be used as non-invasive important biomarkers in lung cancer. Expression of piR-57125 is lower in metastasizing lung adenocarcinoma compared to non-metastatic lung adenocarcinoma (Daugaard et al., 2017). piRL-138 expression has increased with cisplatin therapy in both lung squamous cell carcinoma xenograft models and cells. piRL-138 has increased chemoresistance by suppressing apoptosis in cisplatin therapy for lung squamous cell carcinoma. In addition, suppression of piRL-138 expression has stimulated apoptosis by increasing expressions of PARP and caspase-3 proteins in cisplatin treated xenograft models and cells. piRL-138 reduced cisplatin-induced apoptosis in P53 mutant lung cancer. Thus, targeting piRL-138 may increase the susceptibility of lung cancer to chemotherapeutic agents (Wang et al., 2017).

Prostate cancer

Prostate cancer is the most common type of cancer in men (Siegel et al., 2022). While the five-year survival rate is very high in patients with localized prostate cancer, the patients having metastatic prostate cancer

is approximately 31% (American Cancer Society, 2022). Expressions of piR-002468, piR-158533, piR-349843 and piR-382289 are upregulated in urinary extracellular vesicles of prostate cancer patients compared with healthy controls. In addition, these four piRNAs have binding sites with some mRNAs that have a critical role in the Wnt/beta-catenin, PTEN/PI3K/Akt or androgen receptor pathways, which are important in the pathogenesis of prostate cancer. These results suggest that these piRNAs seems to be potential noninvasive diagnostic markers in prostate cancer (Peng et al., 2021). Expressions of piR-017184 and piR-001773 are also upregulated in prostate cancer tissues. This upregulation has triggered tumor growth both *in vitro* and *in vivo*. Protocadherin 9 (PCDH9) expression is downregulated in prostate cancer and has a tumor suppressive effect. It has been found that PCDH9 exerts a tumor suppressive effect via p85α and Akt. Moreover, piR-017184 and piR-001773 have been shown to regulate PCDH9 at the posttranscriptional level. It has been concluded that these piRNAs exhibit an oncogenic role in prostate cancer (Zhang et al., 2020). piR-19166 expression was found to be lower in both prostate cancer tissues and prostate cancer cells. Transfection of piR-19166 to prostate cancer cells suppressed cell migration by decreasing MT1-MMP, MMP9 and MMP9 expressions. Moreover, piR-19166 has been found to directly target the 3'-UTR of the Cortactin gene. piR-19166 has been thought to suppress migration and metastasis via cortactin and matrix metalloproteinases in prostate cancer (Qi et al., 2020).

Breast cancer

Breast cancer continues to rank second from lung cancer among cancer caused deaths for women in the USA (American Cancer Society, 2022). piR-36712 expression is downregulated in breast cancer compared with non-cancerous breast tissues. piR-36712 has suppressed progression and chemoresistance by inhibiting selenoprotein W1 (SEPW1) in breast cancer. piR-36712 downregulation is associated with poor prognosis. Knockdown of piR-36712 have increased SEPW1 expression. It has been showed that this upregulation can cause downregulated P53, P21 and E-cadherin but Slug upregulation. Therefore, lower piR-36712 expression can increase breast cancer cell proliferation, migration and invasion via these mechanisms. piR-36712 has been found to have anticancer effects synergistically with paclitaxel and doxorubicin in breast cancer (Tan et al., 2019). Variant piR-021285 mimic stimulates invasion by attenuating the 5′-UTR/first exon methylation of proinvasive Rho GTPase Activating Protein 11A (ARHGAP11A) gene in breast cancer. Therefore, piR-012185 suppresses breast tumorigenesis via the ARHGAP11A gene by affecting cancer epigenetic (Fu et al., 2015). piR-36026 has direct-

ly regulated lecithin retinol acyltransferase (LRAT) and serpin family A member 1 (SERPINA1) genes, which have tumor suppressor properties, in breast cancer cells through their piR-36026 binding site. piR-36026 has increased breast cancer cell proliferation via SERPINA1, LRAT and caspase-3 (Lee et al., 2016). piR-932 expression was found to be high and positively regulated breast cancer stem cells by inducing latexin methylation (a tumor suppressor protein). piR-932 may be a therapeutic target for breast cancer (Zhang et al., 2013). piR-20582, piR-20485, piR-4987 and piR-20365 are upregulated in breast cancer tissues compared to non-tumor tissues. piR-4987 has also been related to lymph node metastasis in breast cancer (Huang et al., 2013).

Colon cancer

Colon cancer is among the top five cancer-related deaths and cases in both genders (Siegel et al., 2022). Serum piR-020365, piR-017724, piR-017723, piR-004153 and piR-001311 levels has downregulated in colorectal cancer compared with the healthy donors. Moreover, serum piR-017724 level is positively correlated with survival in patients with colorectal cancer. Results of cox regression analysis have showed that serum piR-017724 level is prognostic factor in patients with colorectal cancer (Qu et al., 2019). The expression of LNC00964-3 containing the piR-015551 sequence is lower in colon cancer tissues compared to normal tissues. However, no significant change in expression of piR-015551 has been observed. piR-015551 expression has been found to be positively correlated with LNC00964-3 expression in colorectal cancer tissues and matched non-cancer tissues. These data indicated that piR-015551 could originate from LNC00964-3, which is a lncRNA and its expression is altered in colon cancer (Chu et al., 2015a). piR-1245 expression has significantly been related advantage stage and metastasis in colorectal cancer. piR-1245 shows oncogenic properties and may cause tumor progression. piR-1245 is the direct target of MDX1, TP53INP1, SESN2, UPP1, NFKBIA, FAS, DUSP1, BTG1 and ATF3 tumor suppressor genes. An inverse correlation between these genes and piR-1245 has been observed in colorectal cancer. Weng et al., (2018) suggested that piR-1245 can be prognostic marker for colorectal cancer. piR-54265 exhibits oncogenic properties and it is thought that it may have therapeutic target potential in colorectal cancer. Expression of piR-54265 is found to be high in colorectal cancer, and its overexpression is positively correlated with poor survival in these cancer patients. piRNA-54265 activates STAT3 signaling pathway by causing the formation of the PIWIL2/STAT3/p-SRC complex. STAT3 signaling pathway also stimulates colorectal cancer cell proliferation, metastasis and chemoresistance. Knockdown of piR-54265

inhibits proliferation and metastasis in mice with colorectal cancer. It has been observed that colorectal cancer patients having low serum piR-54265 levels had better respond to chemotherapy than those with high piR-54265 expression (Mai et al., 2018). piR-28876 and piR-5937 have been thought to be diagnostic biomarkers in colorectal cancer. Because these piRNAs have been found to be low in the serum of patients with colorectal cancer when compared to healthy human blood serum, and they have been reported to have diagnostic potential when investigated only in patients with clinical stage I. In addition, these piRNAs has diagnostic potential for colorectal cancer with higher sensitivity than CEA and CA19-9 markers currently used in diagnosis purposes (Vychytilova-Faltejskova et al., 2018). piR-24000 expression level is higher in colorectal cancer tissues compared to adjacent normal tissues. Also, piR-24000 expression has been found to be positively correlated with metastasis, tumor stage and poor prognosis in colorectal cancer. In addition, ROC analysis has showed that piR-24000 could be a diagnostic biomarker in colorectal cancer (Iyer et al., 2020). The expression level of piR-651 is high in colorectal cancer compared to non-cancerous colon tissues, as in many other cancer types (Cheng et al., 2011).

Pancreatic cancer

Although the survival rate in patients with pancreatic cancer is approximately 11%, it is among the cancers with the lowest survival rate. In addition, pancreatic cancer death rate is increasing day by day (Siegel et al., 2022). piR-017061 expression is significantly low in pancreatic cancer tissues and cells. piR-017061 has been shown to suppress proliferation of pancreatic cancer cell both in vitro and in vivo. It has also been reported that one of piR-017061 targets is Ephrin A5 (EFNA5), which is known to be high in expression in pancreatic ductal adenocarcinomas. Moreover, EFNA5 was accumulated when expression of piR-017061 has suppressed. Overexpression of piR-017061 has resulted in degradation of EFNA5 mRNA. These data suggest that this piRNA may have a therapeutic effect in pancreatic cancer through its tumor-suppressing effect by regulating EFNA5. The therapeutic targeting of EFNA5 is an advantage, as it is a membrane protein (Xie et al., 2021).

Hepatocellular carcinoma

Hepatocellular cancer is one of cancer types with both high incidence and high mortality rate in both women and men in many countries (Korean Liver Cancer Association, 2018). Expression level of piR-Hep1 is higher in hepatocellular carcinoma tissues compared with adjacent non-cancerous liver tissues. Moreover, knockdown of piR-Hep1 has been found to

result in suppression of proliferation, migration, invasion and decreased AKT activation in hepatocellular carcinoma cells (Law et al., 2013). Expressions of piR-00823, piR-LLi-30552, piR-013306 and piR-020498 are high in hepatocellular cancer tissues. As a result of functional analysis, it was seen that these piRNAs can target PTEN, p53, PI3K/Akt and TNF signaling pathways and are critically important in hepatocellular cancer (Rizzo et al., 2016).

Ovarian cancer

Epithelial ovarian cancer accounts for approximately 90% of ovarian cancer cases. Most of these are serous ovarian cancers having the worst prognosis. Ovarian cancer has a 49% five-year survival rate because it is often diagnosed in advanced stages. The five-year survival rate in localized ovarian cancer is 93% because of the more promising response to treatment in the early stages (American Cancer Society, 2022). piR-52207 expression is upregulated in both endometrioid ovarian cancer (ENOCa) tissues and serous ovarian cancer (SOCa) tissues compared to normal ovarian tissue. piR-33733 expression is only higher in SOCa tissues. piR-52207 targets M-phase phosphoprotein 8 (MPHOSPH8), 5-methyltetrahydrofolate-homocysteine methyl transferase (MTR), nudix hydrolase 4 (NUDT4) and eukaryotic translation initiation factor 2, subunit 3 (EIF2S3) in ENOCa. These genes are important in tumorigenesis, proliferation and migration. piR-52207 indirectly regulates D-myo-inositol-5-phosphate, 3-phosphoinositide biosynthesis, vascular endothelial growth factor (VEGF) signaling, EIF2 signaling and folate transformation I by changing the expression of these genes in ENOCa. In addition, piR-33733 has binding site in lipoic acid synthetase (LIAS) gene and piR-52270 has binding sites in actin-related protein 10 homolog (ACTR10) and pleckstrin homology domain containing family A member 5 (PLEKHA5) in serous SOCa. These genes are known to be associated with cell proliferation, reduced apoptosis, migration and tumorigenesis. piR-52207 has regulated lipoate biosynthetase pathway by modulating LIAS gene in SOCa. piR-33733 indirectly regulates the PI3K/Akt pathway via PLEKHA5. piR-33733 also modulates cytoskeletal organization via ACTR10 in SOCa (Singh et al., 2018).

Bladder cancer

Bladder cancer has a five-year survival rate of 77%. Due to its early diagnosis, approximately half of the cases are diagnosed at an early stage, and the five-year survival rate is 96% for those diagnosed at an early stage (American Cancer Society, 2022). Expression of piR-60152 is low in the bladder cancer compared with adjacent normal tissues. Overexpression of

this piRNA can induce apoptosis and suppress proliferation and colony formation in bladder cancer cells. HIWI-piR-60152 complex can affect bladder cancer development by targeting TNFSF4 3'-UTR. Moreover, TNFSF4 expression is downregulated in the bladder cancer compared with adjacent normal tissues (Chu et al., 2015b).

Multiple myeloma

Multiple myeloma is one of the most common hematological cancers. It has approximately 50% five-year survival rate (Kumar et al., 2014). piR-823 expression is high in patients with multiple myeloma and cell lines. piR-823 expression has also been related with clinical stage of patients with multiple myeloma. Knockdown of piR-823 has shown anti-tumorigenic effect by causing cell cycle arrest and apoptosis of multiple myeloma cells *in vitro* and *in vivo*. It has caused cell cycle arrest by inhibiting pRB, cyclinD1 and CDK4 expressions. Suppression of piRNA-823 has been also found to cause inhibition of pAKT and pERK. Knockdown of piR-823 has induced to apoptosis by resulting in decreasing in Bcl-2 and Bcl-xl antiapoptotic genes and proteins, and increasing in expressions of cleaved caspase-3 and poly (ADP-ribose) polymerase. This piRNA suppression also reduced DNMT3A and DNMT3B expression in multiple myeloma cells at both mRNA and protein levels. Moreover, piR-823 expression has been showed to be positively correlated with DNMT3A and DNMT3B in multiple myeloma tissues. piR-823 can directly regulate de novo DNMTs in multiple myeloma cells. It is possible to conclude that piRNA-823 is a direct regulator of *de novo* DNMTs expression in multiple myeloma cells. Inhibition of piR-823 has decreased methylation of tumor suppresor p16 gene. Knockdown of piR-823 has inhibited some angiogenic factors in vitro multiple myeloma cells. These findings suggest that piR-823 plays an oncogenic role in multiple myeloma (Yan et al., 2015). piR-004800 expression is upregulated in patients with multiple myeloma and positively associated with stages of multiple myeloma. piR-004800 knockdown has increased autophagy and apoptosis in multiple myeloma cells. piR-004800 has been shown to regulate the PI3K/Akt/mTOR pathway via the sphingosine-1-phosphate receptor (S1PR). Therefore, it was concluded that piR-004800 has an oncogenic role in multiple myeloma and will be a potential therapeutic target (Ma et al., 2020).

Glioblastoma

Glioblastoma arises from astrocytes and has the most aggressive form among glial tumors. It has a poor prognosis with survival of approximately 14-15 months (Wesseling and Capper 2018). piR-8041 expression is lower in human glioblastoma tissues compared to normal brain tissues.

This piRNA has been shown to repress tumor development in vitro and in vivo. Overexpression of this piRNA has been shown to suppress proliferation and survival pathways and stimulate cell cycle arrest and apoptosis pathways of glioma cells in vitro. Moreover, piR-8041 overexpression resulted in decreased tumor volume in mouse xenograft models (Jacobs et al., 2018). Overexpression of piR-30188 has caused suppression of glioma cell proliferation (Liu et al., 2018).

Expressions of piR-12488, piR-12487, piR-9491 and piR-1849 are also downregulated in glioblastoma patients. piR-9491 and piR-12488 have been shown to suppress the colony forming ability of glioblastoma cells. It has also been reported that piR-23231 is positively associated with survival in patient tissues with glioblastoma (Bartos et al., 2021).

Gastric cancer

Gastric cancer is fifth in incidence and fourth in mortality among cancer types worldwide. It has thereby continued to be an important type of cancer worldwide (Sung et al., 2021). piR-1245 has been reported to be overexpressed in gastric cancer. High piR-1245 expression has been correlated poorer survival TNM stage and size of the tumor in this cancer (Zhou et al., 2020). piR-651 and piR-823 expressions in peripheral blood samples are lower in gastric cancer patients compared with controls. Interestingly, levels of these piRNAs are even lower in postoperative patients. Moreover, PiR-823 has positively been correlated with TNM stage and metastasis (Cui et al., 2011). Other studies also showed that piR-823 expression is lower in gastric cancer compared to normal tissues. Increased expression of piR-823 has suppressed cell growth in gastric cancer cells. The tumor suppressive function of piR-823 has been confirmed in mice experiments. Downregulated expression of piR-823 has been shown to be important in gastric cancer pathogenesis (Cheng et al., 2012). The expression level of piR-651 is high in gastric cancer tissues and correlated with TNM classification. Inhibition of piR-651 has arrested gastric cancer cell cycle in the G2/M phase (Cheng et al., 2011).

Kidney (renal) cancer

Kidney cancer has a high survival rate of 76%, as approximately 66% of patients are diagnosed at an early stage (American Cancer Society, 2022). Expression of piR-57125 has been decreased in metastatic tumors compared with primer tumors in clear cell renal cell cancer. Expressions of piR-30924 and piR-38756 have been increased in metastatic tumors compared to non-metastatic tumors in this cancer. Changes in the expression of these piRNAs have also been associated with tumor recurrence and survival. In addition, Multivariate Cox regression analysis

showed that piR-30924 and piR-57125 may be prognostic biomakers in clear cell renal carcinoma (Bush et al., 2015). piR-823 level is low in renal cell carcinoma tumor tissues compared with adjacent non-tumor tissues. Interestingly, piR-823 level in tumor tissue has been negatively correlated disease-free survival in patients. It has a complex role in renal cell carcinoma. piR-823 expression has been upregulated in serum and urine of these patients compared with healthy controls. However, expression of piR-823 is significantly higher in patients' urine compared to serum. Therefore, urine analysis of piR-823 may be a more promising diagnostic factor in patients with renal cell carcinoma (Iliev et al., 2016). Expression levels of mitochondria-derived piR-51810 and piR-34536 is lower in clear cell renal cell carcinoma (ccRCC) compared with non-cancerous tissues. Downregulation of these piRNAs has been associated with shorter survival of ccRCC patients. It is thought that these piRNAs could be prognostic biomarkers. However, serum piR-51810 and piR-34536 levels are not different in patients compared with healthy control (Zhao et al., 2019). Expression of piR-32051, piR-39894 and piR-43607 are high in ccRCC and correlated tumor stage, metastasis and shorter survival (Li et al., 2015).

Cervical cancer

Cervical cancer remains one of the most common gynecological cancers and fourth most common cancer in women (Bhatla et al., 2021). piR-14633 expression has upregulated in cervical cancer tissues and cells. Elevated piR-14633 expression has induced proliferation, viability, invasion and migration of cervical cancer cells. Moreover, transfection of piR-14633 has increased mRNA stability of methyltransferase-like protein 14 (METTL14) and levels of m6A RNA methylation in cervical cancer cells. PiR-14633 has been shown to directly target 3'-UTR of METTL14. CYP1B1 expression has been found to be increased by overexpression of piR-14633 and decreased by suppression of METTL14. It has also been shown that piR-14633 induces tumor growth in xenograft mice with cervical cancer. As a result, piR-14633 has been found to increase proliferation, invasion and migration via METTL14/CYP1B1 in cervical cancer (Xie et al., 2022).

Conclusion

Cancer consists of somatic cells that proliferate uncontrollably as a result of the disruption of the balance of regulatory mechanisms in normal cells. Cancer stem cells and germ cells have some common mechanisms. When piRNAs were first identified, they are classified as a class of short non-coding RNAs expressed in germ cells. However, studies have shown

that abnormal expression of piRNAs were observed in many cancer types. As studies increase, the molecular mechanisms of many biological functions such as proliferation, cell cycle, apoptosis, invasion and migration have been and continue to be elucidated.

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Chapter 3

DELAYED ONSET MUSCLE SORENESS

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Abstract

Delayed onset muscle soreness (DOMS) is a type of muscle pain that typically occurs a day or two after engaging in physical activity that involves unaccustomed or strenuous muscle contractions. The exact cause of DOMS is not fully understood, but it is thought to be related to microscopic damage to the muscle fibers and surrounding tissue, as well as inflammation in the muscle. There are several strategies that can be used to manage DOMS, including rest, streching, cold and heat modalities, medications, massage and exercise. It is important to note that DOMS is a normal response to physical activity and usually resolves on its own within a few days. However, if the pain persists or is severe, it is important to seek medical attention. In this review, we aimed to compile the most recent studies related to DOMS.

Introduction

Delayed onset muscle soreness, also known as DOMS, is a type of muscle soreness that typically occurs a day or two after engaging in strenuous or unfamiliar exercise (Hotfiel et al., 2018). It is caused by microscopic tears in the muscle fibers and is a normal part of the muscle-building process. Symptoms of DOMS can include stiffness, pain, and reduced range of motion in the affected muscles. The good news is that DOMS is temporary and can be managed with various self-care techniques such as stretching, massage, and icing the affected muscles (Andersen et al., 2013). It is essential to continue to engage in regular physical activity, even if you are experiencing DOMS, as this will help your muscles to recover and become stronger. If you are experiencing severe or persistent muscle soreness, it is always a good idea to consult a healthcare provider to rule out any underlying medical conditions.

DOMS Mechanism

The mechanism of delayed onset muscle soreness (DOMS) is not fully understood, but it is thought to be caused by microscopic tears in the muscle fibers (Armstrong, 1984)doms etiology. When a person engages in strenuous or unfamiliar physical activity, the muscle fibers undergo stress and strain. This can cause tiny tears in the muscle fibers, which leads to inflammation and the release of substances called cytokines. These substances can cause pain and stiffness, leading to the symptoms of DOMS. Over time, the body repairs the damaged muscle fibers, which leads to muscle growth and increased strength.

Several factors can contribute to the development of DOMS, including the intensity and duration of the exercise, the type of exercise being

performed, and the individual's age and gender. Engaging in a new type of exercise or increasing the intensity of a workout can increase the likelihood of experiencing DOMS.

A decrease in the soreness response characterizes delayed onset muscle soreness (DOMS). The specific cause of DOMS is unknown, but several theories attempt to explain it (Armstrong, 1984). One possible model suggests that high tension in the muscle tissue during eccentric exercise leads to structural damage. Cross bridges formed during eccentric actions may also be separated with greater force because the actin-myosin bonds are disrupted before relaxation (STAUBLR, 1989). Second, the damage to the cell membrane can cause disruptions in the levels of calcium within the damaged fibers, leading to necrosis that typically peaks about two days after the initial injury. The third is waste products produced by macrophages. The contents of damaged cells can accumulate in the tissue between cells, stimulating the nerve endings in the muscles and causing DOMS.

The exact cause of DOMS is not well understood, but it is thought to be related to small muscle tears and inflammation that occurs in response to the tears. All theories about the cause of DOMS include (Armstrong, 1984; Cheung, Hume, & Maxwell; Gligoroska & Mancevska, 2021):

1. Microscopic muscle damage: Muscle fibers can be damaged or torn during exercise as they are subjected to heavy loads or unfamiliar movements. This can lead to inflammation and the release of substances called cytokines, which can cause pain and swelling.

2. Lactic acid buildup: It is thought that lactic acid buildup in the muscles during exercise may contribute to DOMS. Lactic acid is a by-product of the breakdown of glucose and is produced during intense exercise when the body's oxygen demand is high.

3. Prolonged muscle tension: Some research suggests that DOMS may be caused by prolonged muscle tension, which can lead to muscle fatigue and damage.

4. Altered muscle metabolism: Exercise can alter muscle metabolism, leading to changes in muscle protein synthesis and breakdown that may contribute to DOMS.

5. High tension in the muscle tissue during eccentric exercise leads to structural damage.

6. The damage to the cell membrane can cause disruptions in the levels of calcium within the damaged fibers, leading to necrosis that typically peaks about two days after the initial injury.

7. Waste products produced by macrophages and the contents of damaged cells can accumulate in the tissue between cells, stimulating the nerve endings in the muscles and causing DOMS.

Overall, the exact cause of DOMS is not fully understood and is likely due to a combination of these and other factors.

DOMS Etiology

Several factors can contribute to the development of delayed onset muscle soreness (DOMS), including(Cheung et al.; Fatouros & Jamurtas, 2016; Gulick & Kimura, 1996):

- They are engaging in strenuous or unfamiliar physical activity. DOMS is most likely to occur after a person engages in a new type of exercise or increases the intensity or duration of their workouts.
- Overuse of muscles. DOMS can also occur when a person uses the same muscles repeatedly without adequate rest and recovery time.
- Age. As people get older, they may be more susceptible to DOMS due to changes in muscle composition and a reduced ability to recover from exercise.
- Gender. Women may be more likely to experience DOMS than men due to differences in muscle composition and hormonal factors.

It is important to note that DOMS is a normal part of the muscle-building process and can occur even in regularly physically active individuals. The key is to listen to your body and give your muscles adequate time to recover after intense exercise.

DOMS and Eccentric Training

Eccentric training, which involves focusing on a movement's eccentric (lowering) phase, can increase muscle soreness and the risk of developing DOMS. This is because eccentric exercises produce more force and stress on the muscles than concentric (lifting) or isometric (static) exercises (Bubbico & Kravitz, 2010). As a result, eccentric training can cause more microscopic tears in the muscle fibers, leading to increased soreness and stiffness. However, it is essential to note that the benefits of eccentric training, such as increased strength and muscle mass, can outweigh the potential for increased muscle soreness. It is important to gradually incorporate eccentric exercises into your workout routine and allow for adequate recovery time to help prevent or minimize DOMS.

DOMS and Injury Risk

DOMS is usually not a serious condition, but it can increase the risk of injury in athletes. One of the main ways that DOMS increases injury risk is by causing functional limitations (Look, Iyengar, Barcellona, & Shortland, 2021). When muscles are sore and stiff, they may not be able to perform at their usual level of strength, flexibility, and range of motion. This can make it difficult for athletes to perform certain movements or activities, increasing the risk of injury. For example, if an athlete has sore leg muscles after a particularly intense workout, they may not be able to jump as high or run as fast as they usually would. This can make them more prone to falls, strains, or other injuries.

Additionally, DOMS can affect an athlete's perception of their abilities. If an athlete's muscles are sore, they may feel weaker or less capable than they actually are. This can lead to a false sense of confidence, which can cause an athlete to push themselves too hard and increase their risk of injury.

To reduce the risk of injury associated with DOMS, athletes must pay attention to their body's signals and listen to their muscles. If muscles are sore, taking a break from high-intensity activities is important and gives the muscles time to recover. Stretching and foam rolling can also help reduce muscle soreness and improve flexibility, which can help prevent injuries (Adamczyk, Gryko, & Boguszewski, 2020).

Overall, while DOMS is a normal part of an exercise and does not usually pose a serious risk to an athlete's health, it is important to be aware of the potential for increased injury risk and take steps to reduce this risk.

DOMS Management and Treatment

Treatment of DOMS typically involves self-care measures such as stretching, massage, and icing the affected muscles (Contrò, Pieretta Mancuso, & Proia, 2016). It is important to continue to engage in regular physical activity, even if you are experiencing DOMS, as this will help your muscles to recover and become stronger.

Several strategies can be used to manage and alleviate DOMS. One of the most effective is to engage in regular low-intensity aerobic exercise, such as walking or swimming (Tufano et al., 2012). This type of exercise can help to increase blood flow to the muscles, which can reduce inflammation and soreness. Stretching and massaging the muscles can also be beneficial, as can the use of ice or heat on the affected area (Krityakiarana, Budworn, Khajohnanan, Suramas, & Puritasang, 2014).

The best way to get rid of DOMS is to allow your body time to recover. This means getting plenty of rest, staying hydrated, and gently stretching the sore muscles. You can also take an over-the-counter pain reliever like ibuprofen or acetaminophen to help reduce pain and inflammation. It is also important to continue exercising, but make sure to give your body time to recover between workouts.

If you do experience DOMS, there are several ways to manage the symptoms and improve recovery (Barata, Cervaens, Resende, Camacho, & Marques, 2011; Ciccone, Leggin, & Callamaro, 1991; Cochrane, 2004; Connolly, Sayers, & McHugh, 2003; Harahap & Siregar, 2021; Hotfiel et al., 2018; K.-J. Kim, Lee, Jung, & Bang, 2009):

1. Rest; taking a break from physical activity can help your muscles recover and reduce the severity of DOMS.

2. Cryotherapy, or the application of cold to the skin, can help reduce inflammation and pain. Cryotherapy may be done using ice packs, cold water immersion, or a cryotherapy chamber.

3. Nonsteroidal anti-inflammatory drugs (NSAIDs); NSAIDs such as ibuprofen and naproxen can help reduce inflammation and pain. It is essential to follow the recommended dosage and duration of use when taking NSAIDs and to talk to a healthcare provider before using them if you have any underlying health conditions or are taking other medications.

4. Gentle stretching; gentle stretching can help improve flexibility and reduce muscle stiffness.

5. Ultrasound; is for promoting inflammatory response while heating tissue and managing blood flow.

6. TENS or analgesic electrical stimulations; for decreasing pain severity

7. Hyperbaric oxygen therapy; can help improve the healing process and promote the growth of new blood vessels.

8. Aerobic exercises; (jogging, cycling, swimming, etc.) for pain relief by increasing endorphin release.

9. Massage; can help improve circulation and reduce muscle soreness. Choosing a massage therapist trained and experienced in working with athletes and muscle soreness is important.

10. Heat; applying heat to the affected muscles can help relax the muscles and reduce stiffness.

11. Compression; wearing compression garments, such as compression socks or sleeves, can help improve circulation and reduce muscle soreness.

12. Stay hydrated; drink plenty of water to help flush out toxins and reduce inflammation.

Overall, while DOMS is a normal part of the exercise, it is important to take steps to minimize the risk and manage the symptoms to ensure that you can continue participating in physical activity.

DOMS and NSAIDS

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a medication often used to reduce inflammation, swelling, and pain. They are commonly used to treat conditions such as arthritis, headaches, and muscle aches, and they are also often used to treat delayed onset muscle soreness (DOMS). NSAIDs work by inhibiting the production of prostaglandins, which are chemicals that contribute to inflammation and pain. By reducing the production of prostaglandins, NSAIDs can help reduce inflammation and pain. Some common NSAIDs that are used to treat DOMS include ibuprofen, naproxen, and aspirin.

Some evidence suggests that NSAIDs may be effective at reducing the severity of DOMS and improving muscle function (Baldwin Lanier, 2004). A review of studies found that NSAIDs may reduce muscle soreness and improve muscle strength in people with DOMS (Contrò et al., 2016). However, the evidence needs to be clarified on the best dosage, duration, or timing of NSAID use for DOMS.

Despite their potential benefits, NSAIDs also have some potential risks and side effects. They can cause gastrointestinal irritation, ulcers, and bleeding and may also increase the risk of cardiovascular events such as heart attacks and strokes. It is essential to follow the recommended dosage and duration of use when taking NSAIDs and to talk to a healthcare provider before using them if you have any underlying health conditions or are taking other medications (Baldwin Lanier, 2004).

Overall, while NSAIDs may be effective at reducing the severity of DOMS and improving muscle function, they should be used with caution and under the guidance of a healthcare provider. Other options for managing DOMS, such as rest, ice, stretching, and foam rolling, may help reduce muscle soreness and improve recovery.

DOMS and Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy (HBOT) is a medical treatment that involves breathing pure oxygen in a pressurized chamber. The pressure in the chamber is greater than the atmospheric pressure outside the chamber, allowing the lungs to take in more oxygen than they would be able to at normal atmospheric pressure. This increased oxygen concentration in the blood can help improve the healing process and promote the growth of new blood vessels.

HBOT is typically used to treat various medical conditions, including carbon monoxide poisoning, decompression sickness, and wounds that are slow to heal due to poor circulation or infection. It is also sometimes used to treat certain types of infections, such as gangrene, and to reduce inflammation and swelling in the brain or spinal cord after a traumatic injury.

HBOT is typically administered in a specialized hyperbaric oxygen chamber or tent, and the treatment lasts one to two hours. It is usually administered on an outpatient basis, although in some cases, it may be administered in a hospital setting. HBOT is generally considered safe, although it can cause side effects such as sinus pain, hearing loss, and temporary vision changes.

Studies have shown that HBOT is effective in returning eccentric force in the quadriceps muscle after DOMS and reducing pain and fatigue (Hart, Tarnopolsky, Lotter, & Babul, 2000; D. J. Kim, Choi, & Son, 2019).

DOMS and Stretching

Stretching is a common technique that can be used to manage delayed onset muscle soreness (DOMS). When you stretch a muscle, you are lengthening the muscle fibers and increasing the range of motion around a joint. This can help to reduce stiffness and improve flexibility, which can help to alleviate the symptoms of DOMS (Xie et al., 2018).

Many types of stretches can effectively manage DOMS, including static, dynamic, and foam rolling. Static stretches involve holding a stretched position for a while, while dynamic stretches involve moving through a range of motion. Foam rolling involves using a cylindrical foam roller to apply pressure to the muscles, which can help to release tension and improve flexibility (Harahap & Siregar, 2021).

It is important to perform stretches properly in order to avoid injury. This means holding each stretch for at least 30 seconds and avoiding bouncing or jerking movements. It is also important to listen to your body and only stretch to the point of mild discomfort.

DOMS and Massage

Massage is a general term for pressing, rubbing, and manipulating the skin, muscles, tendons, and ligaments to improve overall health and well-being. It has many potential benefits, including reducing stress, improving circulation, and relieving muscle soreness and tension.

Massage can be an effective way to relieve the symptoms of DOMS (Visconti, Capra, Carta, Forni, & Janin, 2015). By gently kneading the muscles and increasing blood flow to the area, massage can help reduce inflammation and soreness, making it easier to move and exercise. It can also help relax the muscles and reduce tension, further alleviating soreness (Ernst, 1998).

DOMS and Cryotherapy

The use of ice or heat to treat DOMS is a matter of debate among healthcare providers and fitness experts. Some researchers indicate that using ice on the muscles after a workout can help reduce inflammation and soreness, while others believe that heat is more effective at relaxing the muscles and increasing blood flow to the area (Cochrane, 2004). Cryotherapy can reduce swelling and decrease the rate of metabolism, which in turn reduces the response, vascular permeability, and the formation of edema. However, research to date has not shown that cryotherapy significantly reduces the severity of muscle soreness or speeds up recovery. Therefore, the cold application does not offer much benefit in preventing or treating DOMS, aside from its pain-relieving effect (Nogueira, Felappi, Lima, & Medeiros, 2020). This is in contrast to the effectiveness of cryotherapy in treating acute traumatic injuries, which may suggest that DOMS involves a different or smaller scale of the inflammatory response (Hohenauer, Taeymans, Baeyens, Clarys, & Clijsen, 2015).

There is no definitive answer to this question, and the best approach may vary depending on the individual and the severity of their DOMS. In general, it is important to listen to your body and use whichever method provides the most relief.

DOMS and Heat Modalities

Ultrasound treatment involves using a hand-held device to apply sound waves to the affected area. The sound waves produce heat and create a massage-like effect on the muscles, which can help reduce muscle spasms, stiffness, and pain. Ultrasound treatment is usually performed by a physical therapist or other healthcare providers, and it is typically done in a series of treatments over a few weeks.

There is evidence that ultrasound treatment may effectively reduce the severity of DOMS and improve muscle function (Hotfiel et al., 2018). A review of studies found that ultrasound treatment may reduce muscle soreness and improve muscle strength in people with DOMS (Xia et al., 2022). However, the evidence needs to be clarified on the best frequency, duration, or timing of ultrasound treatment for DOMS (Plaskett, Tiidus, & Livingston, 1999).

Despite its potential benefits, ultrasound treatment also has some potential risks and side effects. It may cause skin irritation or burns if not used properly and may not be suitable for people with certain medical conditions or implants. It is important to follow the recommended dosage and duration of treatment when using ultrasound and to talk to a healthcare provider before starting treatment if you have any underlying health conditions or are taking other medications.

Overall, while ultrasound treatment may be effective at reducing the severity of DOMS and improving muscle function, it should be used with caution and under the guidance of a healthcare provider. Other options for managing DOMS, such as rest, ice, stretching, and foam rolling, may help reduce muscle soreness and improve recovery.

DOMS and Exercise

Aerobic exercises are not typically associated with DOMS. Aerobic exercise involves using large muscles in the body, such as the legs and arms, in a continuous and rhythmic manner (Tufano et al., 2012). Examples of aerobic exercise include running, cycling, swimming, and dancing.

Aerobic exercise is typically less intense than other types, such as resistance or high-intensity interval training (HIIT). As a result, it is not typically associated with the same level of muscle soreness and inflammation as these other forms of exercise (Setiawan, Hikmah, & Agustina, 2022). However, it is important to note that everyone is different, and some people may experience DOMS after aerobic exercise. If you are new to exercise or are increasing the intensity of your workouts, you may be more likely to experience DOMS.

It is important to take steps to minimize the risk of DOMS. Here are some tips for preventing DOMS and reducing its severity (Aiyegbusi, Aturu, & Akinfeleye, 2016; High, Howley, & Franks, 1989; Rodenburg, Steenbeek, Schiereck, & Bär, 1994; Sayers & Dannecker, 2004):

1. Gradually increase the intensity and duration of your workouts: Gradually increasing the intensity and duration of your workouts can help

your muscles adapt to the demands of exercise and reduce the risk of DOMS.

2. Warm-up and cool down: Warming up and cooling down before and after exercise can help your muscles prepare for and recover from the demands of exercise, which can reduce the risk of DOMS.

3. Stretch: Stretching can help improve flexibility and reduce muscle stiffness, which can help prevent DOMS.

4. Use proper form: Using proper form during exercise can help reduce the risk of muscle strain and injury, which can lead to DOMS.

5. Drink plenty of water: Staying hydrated can help prevent muscle fatigue and soreness.

Finally, give your body enough time to recover between workouts. This means getting enough sleep and avoiding working for the same muscle groups on consecutive days.

DOMS and Athletic Performance

DOMS (delayed onset muscle soreness) can affect athletic performance. DOMS typically occurs 24-48 hours after a workout; the effect can last up to a week. Moreover DOMS can cause muscle weakness, stiffness, and reduced range of motion, which can all impact athletic performance (Smith, 1992). When you perform a new or intense workout, your muscles are stressed and can become sore as a result. Microscopic tears in the muscle fibers cause this soreness, and the body's natural response to these tears is to repair and rebuild the muscle. This process can take a week, during which time you may experience muscle weakness, stiffness, and reduced range of motion. These symptoms can impact your athletic performance, particularly if a competition or event is coming up (Sayers & Dannecker, 2004).

An inability to perform an activity or function within the normal range for an individual can be called a functional limitation. This can be significant when a person is experiencing muscle soreness, as their incorrect perception of their temporary impairment may increase their risk of injury (Vasudevan, 1992).

In the studies, it was observed that there were significant decreases in strength and power parameters (Pearcey et al., 2015; Veqar & Imtiyaz, 2014). While these decreases are also seen in isometric and concentric forces, they are most clearly seen in eccentric force measurements. After about a day or two, it can be seen very clearly in the peak torque force.

While isometric and concentric forces return to normal levels in 4-5 days, it may take more than a week for eccentric forces to return.

It is important to allow adequate time for recovery and to use proper stretching and warm-up techniques to help prevent DOMS.

Conclusion

In summary, the mechanism of DOMS involves microscopic tears in the muscle fibers, leading to inflammation and the release of pain-causing substances. While it is a normal part of the muscle-building process, it is important to listen to your body and make sure to give your muscles adequate time to recover after intense exercise (This period may be extended up to 7-10 days).While DOMS is a normal part of the exercise, it is important to take steps to minimize the risk and manage the symptoms to ensure that you can continue participating in physical activity. Exercising while experiencing severe muscle soreness can lead to further injury or overtraining. However, it is important to consult with a healthcare provider or physiotherapist to determine the best approach for relieving DOMS.

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Chapter 4

EFFECTS OF PREBIOTICS ON HUMAN HEALTH

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INTRODUCTION

Healthy is about what human eat in a general term. Thus, daily diet is very crucial. Many bioactive rich nutrition ingredients were predicted as a food waste. Their importance is on increase as the awareness as to the nutrition is on rise. The most favourable human diet for the health should contain some nutrition called as prebiotics, which are a form of dietary fibre that could manipulate human gut microbiota. Consuming prebiotics could enhance content of friendly bacteria available in the gut microbiota. Even though prebiotics could not be digested, this stimulates the growth and the activity of the bacteria being present in the colon. The bacteria were first discovered by Marcel Roberfroid in 1995. Before the discovery, prebiotics were commonly identified as non-digestible food parts.

Common plant-based carbohydrates, mainly oligosaccharides and resistant starch, are the source for the prebiotics. Except for these compounds, pectin, beta-glucans and xylooligosaccharides were also accepted as prebiotic. the mechanism for the healthy effect of prebiotics is related to enhancing mineral absorption, the activity of the microbiota to try digestion of the compounds and even inhibiting the growth and the activity of pathogenic microbes (Langlands et. al., 2004). Even though gut microbiota includes many of different microorganism species, Bifidobacteria and Lactobacillus are the key microorganisms enabling the prebiotics to show the healthy effect on the host by the activity of carbohydrate-modifying enzyme, which could produce short chain fatty acids beneficial for the healthy (Miller et. al., 2009). This chapter is about the claimed health effects of probiotics used in the studies and found that they have healthy effect on human. It has been stated so far that prebiotics in diet could be used for various diseases including intestinal infections in infants, osteoporosis, colon cancer and even inflammatory bowel illness.

Prebiotics are nondigestible food ingredients, which beneficially affect the host not only by selectively stimulating the growth of healthy strains but also by preventing the growth of harmful strains in the intestinal microflora (Gibson & Roberfroid, 1995). Prebiotics, which are currently in use, include inulins, fructo-oligosaccharides (FOS), soya oligosaccharides, xylo-oligosaccharides, lactulose, stachyose, raffinose, lactosucrose, galactooligosaccharides, isomalto-oligosaccharides and palatinose (Lee & Salminen, 2009). One advantage that prebiotics have over probiotics is that the target bacteria already exist in the host (Langlands et. al., 2004).

There are massive evidence showing that prebiotics in food could enhance life quality of human. Infants were stated to be healthier with an appropriate diet (Goldman, 2000; Reuter, 2001). Goldman (2000) and Reuter (2001) mentioned that the diet is one of the important factors in-

fluencing intestinal microflora composition and found that infants fed by their mum had higher number of beneficial bacteria including Bifidobacterium in their gut than the infants commonly consuming baby foods. They assessed that infants consuming baby foods could be more easily to get ill than the infants fed by their mum due to low level of beneficial bacteria, because diet influences the level of Bifidobacterium and Lactobacillus in the intestinal microflora and the higher the level of Bifidobacterium and Lactobacillus in the intestinal microflora, the lower chance infants have any infection (Moro et. al., 2002). The most highly possible reason for why these two groups have different microflora composition could be due to prebiotics in mother milk (Bode, 2009). HMOs (Human Milk Oligosaccharides) are effective against colonization of pathogenic bacteria, since, they are able to inhibit bacterial adhesion to the intestinal, therefore, are preventive the first step of infective process (Kunz & Rudloff, 1993). However, HMOs just exists in human milk and industrial manufacture of HMOs is not possible, thus, Parrett and Edwards (1997) have suggested that other prebiotics such as inulin or, fructo- or galacto- oligosaccharides could be industrially produced for the same purpose. Adam et. al. (2012) have found that prebiotics alone or as symbiotic with probiotics can be utilized for treating the intestinal infections by manipulating the intestinal microbiota. However, more studies in this area should be done to clearly define which prebiotics alone or as symbiotic with probiotics can positively affect the infection diseases in human.

Colon cancer is a serious disease which could be seen due to abnormal cell activity in the colon (Adrouny, 2002). Fiedenreich et. al. (1994) stated that prebiotics are capable of reducing the occurrence of colon cancer. The gut microflora may produce genotoxic and carcinogenic metabolites which play a key role in the start of the disease (Rowland and Gangolli, 1999; Venturi et. al., 1997). Carcinogen metabolites such as radiactive carcinoges (gamma rays or alpha particles) or non- radioactive carcinoges (tobacco, certain dioxins) are important to consider, because they can cause cancer due to the ability to damage the genome (DNA directly in cells) or to the disruption of cellular metabolic functions. These may cause an uncontrolled cell division in host (Ali et. al., 2003). However, Burns and Rowland (2000) hypothesized that increasing the consumption of prebiotics in diet could prevent gut microflora from producing these metabolites by positively modulating gut microflora. Hughes and Rowland (2001) analyzed the hypothesis in the study. They used two prebiotic sources, which are fructans-oligofructose (RaftiloseP95) and long chain inulin (RaftilineHP) for eighteen male Sprague–Dawley rats. They found that prebiotic feeding has a protective effect on colon cancer in rats, with having no significant difference between two oligosaccharide

diets. The possible reason for protective effect of prebiotic feeding may be due to increased level of Bifidobacterium in gut microflora (Gibson and Roberfroid, 1995), which may positively affect lipid metabolism and the result of Hughes and Rowland (2001) supports thr possibility, because no significant on bacterial enzyme activity was found when lower ammonia and increased β-glucosidase activity were found (Hughes and Rowland, 2001). The production of SCFAs increases with the increase in the level of Bifidobacterium, SCFAs are able to lower ammonia and increase β-glucosidase activity (Pan et. al., 2009). Rowland et. al. (1998) stated that inulin diet itself is effective at reducing the occurrence of colon cancer caused by azoxymethane in rats and the combined diet (inulin + Bifidobacterium longum 25) is more effective at colon cancer than that of prebiotic feeding alone. It may be due to host factors or symbiotic factors of prebiotics and probiotics (both affected the same target). More studies are needed to investigate these findings in human subjects to clearly identify the efficiency of the symbiotic diet for the human health.

Osteoporosis is a disease due to low bone density. Bone density could be on decrease as bones absorb minerals at a lower rate. Roberfroid (2000) found that prebiotics are able to increase mineral absorption by modulating the intestinal microflora. Katharina & Schrezenmeir (2007) explained the mechanism of how prebiotics can increase mineral absorption stating that prebiotics are able to increase acidity by short-chain fatty acids, particularly butyrate (Trinidad et. al., 1996), which increases solubility of minerals in the gut, with increasing the absorption surface of the gut. Scholz-Ahrens and Schrezenmeir (2002) observed that prebiotics cause an improvement of mineral level due to reduced pH and increased some metabolites of the intestinal microflora. These results should be confirmed in human studies as well, however, an improvement of mineral level in human is harder to observe than that in animals, because humans need some necessary nutrients including vitamins, minerals and essential fatty acids (Scholz-Ahrens and Schrezenmeir, 2002). The possible reason for why an improvement of mineral level in human is tricky, because the human needs a daily adequate intake of these necessary nutrients to the body and when the body couldn't satisfy its nutrition need from the diet, the body begin to use the nutrition available in cells or muscles. Hence, an absolute observation of improvement of mineral level in human couldn't be made. Nevertheless, the study of Griffin et. al. (2002) has stated improvement of mineral uptake in human and shown that prebiotic feeding may be used for the treatment of the diseases of the musculoskeletal system in human. Further studies are needed to investigate connection between long-lasting prebiotic use and increased bone density in humans. Prebiotics have been

extensively used in human diet to observe the effect of prebiotic diet. The effects found in the studies were given at table 1;

Area	Prebiotic	Study details	Main findings	Reference
Cancer	A symbiotic of prebiotic (oligofructose-enriched inulin) and probiotic (Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12) prebiotic used combines 2 different chain structure, average DP of the first was 4 (Highly soluble in water), another`s average was 25 (hardly soluble in water) probiotics dose was 1010 CFU /gram in a 12 gram sachet. The treatment lasted 12 weeks.	37 colon cancer patients were used as subject. The study was a randomized, double-blind, placebo-controlled trial. Fecal and blood samples were obtained before, during, and after the intervention, and colorectal biopsy samples were obtained before and after the intervention. The standard plate count techniques were used to perform fecal flora analysis.	Yhe consumption of the synbiotic positively affected colon cancer in subjects. Moreover, the synbiotic used was able to change fecal flora (Increased level of bi fidobacterium, lactobacillus and decreased level of Clostridium perfringens) and the synbiotic used increased production of interferon γ, which has a macrophage activating effect.	Rafter et. al. (2007)
	A symbiotic of prebiotic (inulin enriched with oligofructose-10% of the diet) and probiotic (Bifidobacterium lactis Bb12 and Lactobacillus rhamnosus each at 5×108 CFU /gram diet) (Diet dosage was not identified). The analysis lasted till the rats die. (31 weeks)	64 male F344 rats (32 for control, 32 for the symbiotic) were used. Rats were fed prepared diet starting 10 days before administration of azoxymethane (15 milligram/kilogram of rat two times daily) until death. At death, analysis of SCFAs was performed by utilizing caecal content collected in gas chromatography and flame ionization detection. It was a controlled and double-blind study.	Prebiotic administration in the diet decreases the occurrence of azoxymethane-induced cancer in rats. Rat group fed with the symbiotic had higher caecal short-chain fatty acids ($p< 0.001$). I assume that SCFAs have a role in cancer from these findings.	Femia et. al. (2002)

Area	Prebiotic	Study details	Main findings	Reference
Cancer	Prebiotic was not identified. The treatment lasted 45 weeks. (Rats were killed by CO2 asphyxiation at week 45)	Fisher 344 male 21 days old rats (n:90) were randomized to 9 groups. Rats are fed 9 different diets: AIN-93G/M (Specified by the author of the study) as control and treatment groups with prebiotics (5%), and the rest 8 group received different combination of prebiotic and soybean meal (5 – 10% for both). Injections of AOM were administered to rats at 7 and 8 weeks when they were 16 milligram/ kilogram body weight.	Prebiotic feeding and soybean, when fed together, reduced the accruing of azoxymethane-induced colon cancer in rats.	Gourineni et. al. (2011)
	There were 6 treatment groups. In prebiotic group, rats are fed with an inulin-based fiber or a novel fiber (polydextrose) at 5% of the diet. (Dosage of the diet was not identified).	The treatment lasted 4 weeks. 5 months old ovariectomized Sprague Dawley rats were used. For each diet, calcium and magnesium metabolic balances of subjects were performed.	Rats receiving polydextrose had significantly higher calcium absorption capacity and retention than all other groups and they have a trend ($P \leq 0.10$) for higher calcium absorption compared to inulin-based fibers but the advantage (higher calcium absorption) had not lasted long time (finished after first explosion). The prebiotic feeding increased calcium metabolism that might be related to changes in the gut (production of SCFAs).	Legette et. al. (2012)

Area	Prebiotic	Study details	Main findings	Reference
Osteoporosis	There were 4treatments received by subjects (15 gram/day of inulin, fructooligosaccharide, or galactooligosaccharide, or not supplemented (Control treatment)).	12 non-anemic young healthy, male subjects aged 20−30 years were used as subject. A double stable-isotope techniques (oral 57Fe and 44Ca and intravenous 58Fe and 48Ca) were used. Iron absorption was analyzed over the last 7 days of treatment (days 15 to 21) when calcium absorption was analyzed on the day 21 of each treatment group. Treatment lasted 21 days for each group.	None of the differences between treatment methods was significant. However, it was observed that 15 gram/day of inulin, fructooligosaccharide, or galactooligosaccharide have a positive effect on iron and calcium absorption in young healthy men.	Dokkum et. al. (1998)
	Short chain fructooligosaccharides (scFOSs) at 10-gram daily dose for 5 weeks. Subjects orally received 44Ca (stable isotope).	12 healthy, postmenopausal women participated the study. A randomized, double-blind crossover stable isotope study was used. The treatments were separated by a 3-week-washout period. The subjects divided into 2 groups according to menopause duration: those who had been menopausal for 2–6 years (n = 6) and those who had been menopausal for > 6 years (n = 6).	It was first observed that scFOSs had no positive effect on calcium absorption in early postmenopausal phase when scFOSs had positive effect in late postmenopausal phase. So, that is why subjects are divided into 2 groups later in the study.	Tahiri et. al. (2003)

Area	Prebiotic	Study details	Main findings	Reference
Inflammatory bowel disease (IBD)	Human milk and a formula diet fed by subjects for 20 days. The dosage of any of treatment was not identified.	Six breast-fed and six formula-fed newborn infants were used as subject. Six fecal samples of each infant were obtained during the first 20 days of their life. The microbial compositions of the samples were analyzed by culturing on specific media and by FISH (independent fluorescent in situ hybridization). The colonies growing on the media were identified by random amplified polymorphic DNA pattern analysis and by polymerase chain reaction amplification.	The development of intestinal flora between breast-fed and formula-fed infants showed differences. The minor components of the fecal samples from breast-fed infants were lactobacillius and streptococcus; samples from formula-fed infants contained staphylococcus, escherichia coli, and clostridia.	Harmsen et. al. (2000)
	The trial consists of two different groups. The group (n = 7) as control took a baseline anti-inflammatory therapy during 4 weeks. Germinated barley foodstuff (GBF-prebiotic feeding) -treated patients (n:11) received 20-30-gram GBF daily, together with the baseline treatment, for 4 weeks.	Eighteen patients with mildly to moderately active UC were used. The response to the treatments and fecal microflora was analyzed clinically.	Oral therapy of a new prebiotic from germinated barley reduced clinical activity of UC in subject ($P < 0.05$), comparing with the control group.	Kanauchi et. al. (2002)

Area	Prebiotic	Study details	Main findings	Reference
Inflammatory bowel disease (IBD)	There were three groups. The probiotic group ingested one daily capsule consisting of Bifidobacterium longum (2 × 109 CFU), the prebiotic group ingested daily 8.0-gram dose of psyllium and the last group received the symbiotic containing prebiotic and probiotic.	94 subjects (Thirty-one subjects of which in the probiotic group, another 31 in the prebiotic group, and the last 32 in the symbiotic group) who were aged 24–67 years and who had not received antibiotics in the last three months) were used in this study. All patients completed Inflammatory Bowel Disease Questionnaires (IBDQs) at the start of the trial, at midpoint (second week) of the trial, and at end (fourth week) of the trial. Blood samples were also analyzed from 32 patients randomly selected from all groups then all the results of blood samples were compared with IBDQ scores.	Symbiotic treatment showed better effect than other groups on treating UC. It may be due to the fact that symbiotic therapy may have a synergistic effect on the treatment of UC.	Fujimori et. al. (2009)
	Subjects received a symbiotic, which contained 2×1011 freeze dried viable Bifidobacterium longum in a gelatin capsule and a sachet containing 6 g of prebiotic fructo-oligosaccharide/ inulin mix for one month.	18 patients with UC were used. A double blinded randomized controlled trial was utilized. Clinical status was scored and rectal biopsies were collected before and after treatment were analyzed.	Short term administration of symbiotic resulted an improvement on the treatment of chronic inflammation in subjects.	Furrie et. al. (2005)

Area	Prebiotic	Study details	Main findings	Reference
Allergic Disorders	Lactobacillus salivarius 433118 (109 CFU/milliliter for 19 weeks) and Bifidobacterium infantis 35624 (108 CFU /milliliter for 19 weeks) in 4–7 milliliter of milk per day.	N = 30 IL-10 KO mice whose ages were 7–9 weeks and a double blind, placebo- controlled trial was used.	Both Lactobacillus salivarius 433118 and Bifidobacterium infantis 35624 are effective at treating allergic disorder ($p<0.05$), with comparing to the placebo group.	McCarthy et. al. (2003)
	Bifidobacterium Lactis Bb-12 (1 x 109 CFU/gram) and Lactobacillus acidophilus ATCC 53103 (3 x 108 CFU / gram). Treatment duration and dosage were not identified.	A total of 27 infants with mean age of 4.6 months. It was a randomized, double blind, placebo-controlled study.	These probiotics can modulate gut microflora, which positively affects allergic disorders.	Isolauri et. al. (2000)
	Lactobacillus fermentum VRI-033 PCC (1×109 CFU /gram twice daily for 8 weeks) 16 weeks is the duration of study. Administration of probiotic was done with 5–10 milliliter of water.	Forth two children aged 6-18 months with moderate or severe AD. (Half of them consumed probiotic when the other half used placebo). It was a randomized, placebo-controlled, cross-over trial.	Probiotic feeding is effective at reducing the severity of atopic dermatitis ($p < 0.05$) with comparing to the placebo group.	Prescott et. al. (2005)

Area	Prebiotic	Study details	Main findings	Reference
Infection Diseases and HMO	The mothers fed the infants in their first 3 month (human feeding or formulated feeding). Any dosage was not identified.	A total of 146 infants were exclusively and 38 infants were partially breast-fed, and 23 infants were exclusively formula - fed (all the infants were 3-month-old). Oral swabs from randomly chosen 73 infants were analyzed by the Human Oral Microbe Identification Microarray (HOMIM) and by quantitative polymerase chain reaction for Lactobacillus gasseri.	The microbiota of the mouth differs between 3- month-old breast-fed and formula -fed infants. Possible reasons for why breast-fed infants have a different mouth microbiota may be due to species in breast milk.	Holgerson et. al. (2013)
	The mothers fed the infants for 2 years. Any dosage was not identified.	93 breast-feeding infants were used as subject. Data was collected during 1988 - 1991 from birth to 2 years with infant feeding weekly. Data was analyzed by poisson regression.	Human milk oligosaccharides have a protection effect on infant diseases.	Morrow et. al. (2004)

Inflammatory Bowel Diseases (IBD) describes a group of various diseases which occur due to inflammatory condition in the small intestine or colon (Stephen and Bloomfeld, 2011). Onderdonk et. al. (1981) demonstrated that ulcerative colitis (UC) occurs in strictly anaerobic gut microflora, later, Langlands et. al. (2004) and Akira et. al. (2013) stated that prebiotic feeding may well prevent or even treat IBD (with manipulating gut microflora). There are three possible reasons for how prebiotic feeding may prevent or even treat IBD in human. First possible reason is related to Bifidobacterium level in gut microflora. Lindsay et. al. (2006) used ten subjects with active ileocolonic Crohn's disease, a fructo-oligosaccharides (F0S) randomized treatment (in which subjects receive 15 g FOS for 3 weeks) and fluorescence in situ hybridisation to qualify faecal

bifidobacterium level. They found that prebiotic feeding has a protective effect on Crohn`s disease in subjects and faecal bifidobacterium level increased in subjects ($p<0.001$) (from 8.8 log10 to 9.4 log10 cells/g dry faeces). Okada et. al. (2009) stated that increase in bifidobacterium level can increase the level of IL-10 (Interleukin-10), which is an anti-inflammatory cytokine. Hence, this may be why an increase in the level of faecal bifidobacterium in 10 subjects of Lindsay et. al. (2006) study show a protective effect (namely due to increased level of IL-10). Second possible reason for how prebiotic feeding can treat or prevent IBD was explained by Gibson et. al. (1991). Gibson et. al. (1991) have demonstrated that the ions in the intestinal microflora could cause diseases in human. They found that sulphate reducing bacteria (SRB) level in health human is 50%, however, this level increases to 100% in IBD patients. SRB may highly possibly cause IBD, because they can produce H2S (Hydrogen sulfide) from the sulphates available in the gut. H2S is cytotoxic to the cells in human and its toxicity can enter to the cells of the colonic mucosa. The cytotoxic of H2S can contribute to the pathogenesis of UC, because H2S reduces the protective function of cells (even causes the death of cells) and the effectiveness of immune system (Ng and Tonzetich, 1984). Third possible reason is that Sorokin et. al. (2010) found that butyrate isolates SRBs activity, therefore, I assumed that butyrate may play a key role in the treatment or even prevention of IBD, because, SRB and butyrate produce bacteria may compete for hydrogen available in the bowel and when butyrate produce bacteria can use more the hydrogen ions, SRB cannot find the hydrogen ions enough to effectively work, then, SRB activity begins to fall (Schmidt and Ahring, 1993). I can suggest prebiotic feeding to increase the production of butyrate by bacteria in the bowel for treatment or prevention of IBD, because Bamba et. al. (2002) found a new prebiotic from germinated barley and this prebiotic is noted in an animal study conducted by Araki et. al., 2000 to have a promoting effect on production of butyrate by acting as a bulking agent and decreasing occurrence of diarrhea (which leads to a increase in production of butyrate). Further studies are performed with human subjects to identify the efficiency of the new prebiotic in SCFAs (especially in production of butyrate).

CONCLUSION

In conclusion, it was discussed about claimed health effects of prebiotic feeding, with special interest of the role of intestinal microflora. Even though probiotics, generally the strains of bifidobacterium or lactobacillus, are alone utilized in the studies, prebiotic trials were done with a symbiotic of probiotic. Even though a symbiotic treatment is the only way to show prebiotic effect in the host, there is a need for prebiotic

feeding-based trials in human, because prebiotic feeding itself can have a healthy effect in the host, with modifying intestinal microflora or influencing enzymes with the production of SCFAs. More studies are needed to investigate of possible healthy benefit from each strain of the prebiotics. Main finding of prebiotic studies, however, shows that a symbiotic has a better health effect than either alone probiotic or prebiotic feeding. It was assumed that it may be due to the symbiotic effect of these two feeding in the host.

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Chapter 5

MARINE-DERIVED FUNGUS *ASPERGILLUS NIGER* IN NEW DRUG SEARCH

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1.1 INTRODUCTION

Along with the progress of history, humanity, the diseases that humanity is connected to, and the methods of combating these diseases have changed. Since drugs, which are the most important weapons in the treatment of diseases, are tolerable for some diseases, their effectiveness has begun to be insufficient for those diseases. For this reason, the discovery of new drugs and these drug sources has gained seriousness. With its easy accessibility, the most researched resources have been plants, animals, and microorganisms that are inhabited in terrestrial. The marine culture is an important resource whose discovery is overdue for new searches and is investigated increasingly for explories of new active substances.

The marine ecology consists of marine creatures containing marine plants, vertebrates, and invertebrates that resist the marine environment's extreme conditions are oligotrophic or osmotic stress, high salt concentrations, excessive pH, lack of oxygen and light, intense radiation, high metal concentrations, and exposure to high pressure are all non-mesophilic (warm or cold) conditions (Giddings & Newman, 2019). In particular, among the marine microorganisms, marine fungi survive in symbiotic relationships with these marine creatures and also which are separated from marine inanimate surroundings such as deep sea, hydrothermal vents, polar systems, mud, mudflat, sediment, and seawater. These marine fungi are exhibited the highest-value active components under these dire circumstances (Saravanakumar et al., 2020). These marine microorganisms are important due to can be safely preserved in the laboratory environment and can be easily cultured and used over and over again.

Marine fungi studies on the pharmaceutical pipeline, which are started with cephalosporin C which is an antibiotic, is obtained from the marine fungus *Cephalosporium acremonium* is secluded from the ocean and next to a sewage channel in Cagliari, Sardinia (Brotzu, 1948), have been continued increasingly. Lastly, belong to marine-derived fungus *Aspergillus* sp., Plinabulin which is investigated as a first-in-class selective immunomodulating microtubule-binding drug (SIMBA) with potential anticancer and prevention of chemotherapy-induced neutropenia (CIN) advantages, has accessed to phase III on clinical trials by the company is BeyondSpring Pharmaceuticals (Kashyap et al.,2019; La Sala et al., 2019). In addition, marine fungi, which are generally evaluated in terms of production of new types of secondary metabolites in biotechnology, are also producing source for lipids, carbohydrates, pigments, enzymes, and vitamins (Damare et al., 2012).

Aspergillus niger (Tieghem, 1867) which is a species from the Nigri section of the genus *Aspergillus*, is a cosmopolitan asexual saprophyte

that contains around the black conidial head with asexual spores and a thin stalk, occurring in all aerobic environments (Bennett, 2010; Varga et al., 2011). *A. niger* is found in several habitats of nature; soil (13%), endophyte (27%), marine (24%), mutant (10%), and others (26%). Fungal strains in these habitats produced secondary metabolites which are had several chemical skeletons such as naphtho-γ-pyrones, α-pyrones, yanutones, cyclopeptides, pyranonigrins, diketopiperazines, itaconic acid derivatives, alkaloids, terpenes, steroids, azaphilones, bicumarins, pigments, sphingolipids and acids. After the first studies associated production of citric acid, via advancing biotechnological and analytical techniques, a wide variety of beneficial proteins which are not occured by other filamentous fungi but are isolated from *A. niger*. Thus, the production of many extracellular enzymes including α-amylase, cellulase, catalase, dehydrogenase, oxidase, hydrolase, pectinase is contributed significantly. (Lima et al., 2019; Rateb and Ebel, 2011). The compounds are obtained from strains of marine *A. niger* showed particularly activities of cytotoxic, antiproliferative, anticancer, antioxidant, antibacterial, insecticidal, antifungal, antiviral, and also rarely bioactivities of enzyme inhibition, and neuroprotective.

In this paper, the isolated secondary metabolites of *Aspergillus niger* which is the marine-derived fungus, between 1997 and the first half of 2022 are indexed with their chemical groups under their activity titles.

2. ACTIVE METABOLITES

2.1. Antibacterial Metabolites

The new compound in farnesylated epoxy cyclohexenone structure, Yanuthone A was isolated from *Aspergillus niger* (F97S11 strain) which inhabited an orange *Aplidium* sp. ascidian picked up from the seas neighboring Caesar's Rock in Benga, Fiji. The isolated compound was examined for their antimicrobial activities on microorganisms which are *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *Staphylococcus aureus* (MSSA), vancomycin-resistant *Enterococcus* by the standard diffusion method. As a result of the inhibition area measurement, Yanuthone A showed minimal activity on MSSA with a worth of 8 mm/175 μg (Bugni et al., 2000).

Malformin A_1 and malformin C are cyclopentapeptide derivatives were isolated from *Aspergillus niger* (MA-132 strain) acquired from a fresh and healthy specimen of the *Avicennia marina* sea mangrove tree inhabited in Dongzhai Port, Hainan, China. Their antibacterial effects were tested against *S. aureus* and *E. coli* bacteria with chloramphenicol (positive control). At 20 μg/disc concentration, chloramphenicol showed

inhibitory activity with inhibition area of 20 mm, while malformin A_1 and malformin C shown little antibacterial activity with inhibiting regions of 9.0 and 8.5 mm, respectively (Liu et al., 2013).

Three new itaconic acid derivatives asperitaconic acid A, B, and C compounds were isolated from *Aspergillus niger* (LS11 strain) obtained from *Haliclona* sp. sponge collected from Linshui, Hainan province, China. All compounds were examined for their antibacterial activity against *S. aureus* bacteria by microdilution method on 96-well microplates. Chloramphenicol which is positive control, had a MIC of 4 μg/mL, whereas compounds of asperitaconic acid A, B, and C showed MIC values of 16, 32, and 32 μg/mL, respectively. All compounds showed weak or moderate antibacterial activity (Ding et al., 2018). Furthermore, derivatives of 4-hydroxy-α-pyrone, new nipyrone A, nipyrone B, and nipyrone C compounds, besides with the known germicidin C compound were acquired from *Aspergillus niger* (LS24 strain) inhabited in the same sponge. The effectiveness of all compounds against *S. aureus*, *B. subtilis*, MRSA, and *E. coli* bacteria was investigated by microdilution method using chloramphenicol as a positive control. Nipyrone C compound showed a strong antibacterial effect against *S. aureus* and *B. subtilis* bacteria with MIC values of 8 and 16 μg/mL, respectively, compared with 8 and 2 μg/mL MIC value of chloramphenicol, respectively. While other compounds showed moderate antibiotic activity against *S. aureus*, *E. coli*, and *B. subtilis* bacteria with MIC values in the range of 32-64 μg/mL, they showed little antibiotic effect against MRSA bacteria with MIC worth of 128 μg/mL. When the antituberculosis activities of all compounds were examined against *Mycobacterium tuberculosis* H37Rv bacteria using ethambutol as a positive control, the nipyrone C compound has a moderate antituberculosis effect with a MIC worth of 64 μg/mL compared to the 8 μg/mL MIC value of ethambutol (Ding et al., 2019).

Fonsecinone A and isoaurasperone A compounds, dimeric naphtho-γ-pyrone derivatives, were obtained from *Aspergillus niger* (L14 strain) acquired from *Reniera japonica* fresh sponge collected from Xinghai Bay, Dalian, China. The antimicrobial efficiency of all substances were assessed by microdilution using the antibiotics metronidazole, clarithromycin, and levofloxacin as positive controls against the human pathogen *Helicobacter pylori* G27 strain and the clinical multidrug-resistant *Helicobacter pylori* 159 strain. Metronidazole, clarithromycin and levofloxacin antibiotics against *H. pylori* G27 strain showed MIC worths of 2, 0.004 and 0.5 μg/mL, respectively, while fonsecinone A and isoaurasperone A compounds showed MIC worths of 2 and 2 μg/mL, respectively. Metronidazole, clarithromycin and levofloxacin antibiotics against *H. pylori* 159 strain showed MIC worths of 16, 2 and 8 μg/mL, respectively, while

fonsecinone A and isoaurasperone A compounds showed MIC worths of 2 and 4 μg/mL, respectively. These compounds have been shown to exert notable inhibitor activities on the human pathogen bacteria *H. pylori* G27 and 159, with MIC worths of ≤4 μg/mL comporable to those of ampicillin sodium as a positive control (Liu et al., 2021). In addition, biosynthetic analysis of bioactive substances was performed with the genomic and AntiSMASH analysis study on this fungal strainThis led to the discovery of 69 secondary metabolite biosynthetic gene clusters in all, including 17 polyketides synthases, 18 non-ribosomal peptide synthases, 21 non-ribosomal peptide synthases-likes, 9 terpenes, 2 indoles, 1 beta lactone, and 1 siderophore (Wang et al., 2022).

The activities of RF-3192C and orlandin compounds which were isolated from the *Aspergillus niger* (ASSB4 strain) obtained from *Laurencia obtuse* red algae lived in Nabq Bay, Red Sea, Egypt, were investigated against *Pseudomonas aeruginosa*, *S. aureus* and *B. subtilis* bacteria by agar disc diffusion method using gentamicin as a positive control. On these bacteria, gentamicin showed 18, 20 and 16 mm zones, respectively, while RF-3192C compound showed high activity with 15, 7 and 14 mm zones, respectively, and orlandin showed high activity with 12 mm zone only on *B. subtilis* (Manar et al., 2021).

2.2. Antifungal Metabolites

Naphtho-γ-pyrone derived compounds, nigerasperone C, aurasperone B, asperpyrone C and fonsecinone A were acquired from *Aspergillus niger* (EN-13 strain) isolated from *Colpomenia sinuosa* brown algae resides in the coastline of Qingdao, China. The antimicrobial activities of the compounds were investigated on *C. albicans* and *A. niger* strains using the agar well diffusion method with amphotericin B as a positive control. Against to *C. albicans,* the clear inhibition zones of amphotericin B (12 mm), nigerasperone C (9 mm), aurasperone B (10 mm), asperpirone C (14 mm) and fonsecinone A (9 mm) compounds demonstrated their weak antifungal activities (Zhang et al., 2007a). In another article published by the same working team, 5,7-dihydroxy-2-[1-(4-methoxy-6-oxo-6H-pyran-2-yl)-2-phenylethylamino]-[1,4]naphthoquinone which a novel naphthoquinoneimine derivative, was obtained from *Aspergillus niger* (EN-13 strain) isolated from *C. sinuosa* brown algae. Using the agar well diffusion technique, the compound exhibited moderate antifungal activity against *C. albicans* with an inhibitory zone (10 mm) at 20 mg/well (6 mm) (Zhang et al., 2007b). Moreover, asperamide A (sphingolipid) which was isolated from *Aspergillus niger* (EN-13 strain), was also examined against *C. albicans*. Asperamide A was displayed moderate activity with a obvi-

ous inhibitory zone (12 mm) compared to the apparent inhibitory zone (12 mm) of the positive control amphotericin B (Zhang et al., 2007c).

2.3. Antiviral Metabolites

The internal ribosomal entry site (IRES) is in the 5'-NTR (5' untranslated region) region containing the translation promoting cis-acting elements of viral mRNA conserved by flaviviruses and picornaviruses. This nucleotide segment responsible for initiating the synthesis of viral polyproteins is an important molecular target for the discovery of medicines that prevent viral translation without affecting the production of protein in the host cell. In this context, TMC-256A1 was isolated from *Aspergillus niger* is gathered by the Australian Institute of Marine Sciences (AIMS) in Townsville, was investigated. The drug's efficiency against viral IRES-mediated translation and its selectivity for viral as opposed to mammalian Cap-dependent translation (Cap) were also explored. The compound TMC-256A1 displayed activity against IRES with IC_{50} worths of 44 and 80 μM on IRES and Cap, respectively (Ovenden et al., 2004).

Aflatoxines, averufin and nidurufin were isolated from *Aspergillus niger* (MF-16 strain) acquired from ocean is procured from Quanzhou Gulf, Fujian province, China. Their inhibitor efficiencies were examined against the tobacco mosaic virus (TMV, plant pathogen) by in vitro leaf-diffusion technique. Averufin and nidurufin showed moderate inhibitory on TMV replication with EC_{50} worth of 0.10 and 0.086 mg/mL, respectively (Wu et al., 2008).

Aspernigrin A and aspernigrin C containing 2-benzylpyridin-4-one metabolite together with known malformin C, rubrofusarin substances were isolated from *Aspergillus niger* (SCSIO Jcsw6F30 strain) acquired from *Sargassum* sp. brown algae was gathered near Yongxing Island, South China Sea. All obtained compounds were examined for their inhibitor efficiencies against infection by chemokine receptor subtype 5 (CCR5) tropic HIV-1(human immunodeficiency virus-1) SF162 on TZM-bl cells (HeLa human cervical carcinoma cells) using the colorimetric XTT method. Against HIV-1 SF162-induced infection, with the IC_{50} worth of 1.4 ± 0.06 μM (selectivity index 11.4), malformin C showed potent antiviral efficiency similar to abacavir (IC_{50} = 0.8 ± 0.1 μM) which is a nucleoside reverse transcriptase inhibitor, and ADS-J1 (IC_{50} = 1.8 ± 0.3 μM) which is an effective HIV-1 entry inhibitor, while aspernigrin C displayed effectively inhibition activity with IC_{50} value of 4.7 ± 0.4 μM (selectivity index 7.5). The antiviral efficiency of aspernigrin A and rubrofusarin were poor, with IC_{50} worth 83.2 ± 21.4 μM and 56.2 ± 3.3 μM, respectively (Zhou et al., 2016).

2.4. Antioxidant Metabolites

Naphtho-γ-pyrone derivative compounds, nigerasperone C, aurasperone B, fonsecinone B, fonsecinone D were isolated from *Aspergillus niger* (EN-13 strain) related with *Colpomenia sinuosa* brown algae Isolated components were examined impact on scavenging DPPH (2,2-Diphenyl-1-picrylhydrazyl) radicals. At 50 µg/mL concentration, nigerasperone C, aurasperone B, fonsecinone B, fonsecinone D were displayed 41.6%, 48.1%, 13.2%, 37.5% values, respectively, compared with 80.4% efficacy of the BHT (butylhydroxytoluene) positive control. According to this results, nigerasperone C and aurasperone B were considered to have moderate antioxidant effects (Zhang et al., 2007a).

By adding NaBr and $CaBr_2$ metal bromides to each 50 mM during the fermentation of the fungus, a new brominated naphthopyranone, 6,9-dibromoflavasperone compound was together with known flavasperone, TMC-256A1, fonsecin (naphtho-γ-pyranone monomers), and aurasperone B (naphtho-γ-pyranone dimer) was isolated from *Aspergillus niger* (MSA773 strain) fungus obtained from sea mudflat collected in Suncheon Bay, Jeonnam province, Korea. All substances were examined for their radical scavenging effect of DPPH with using ascorbic acid as a positive control. Aurasperone B, TMC-256A1, 6.9-dibromoflavasperone, and flavasperone, showed strong antioxidant efficiency with IC_{50} worths 21, 25, 0.3, 0.02, and 0.01 µM, respectively against 20 µM IC_{50} worth of ascorbic acid (Leutou et al., 2016).

Nigerin and ochracene J, the new sesquiterpenoids, were isolated from *Aspergillus niger* (164117 strain) obtained from the internal tissues of *Dysidea* sp., a sea sponge which taken from the Xisha Islands in the South China Sea. Nitric oxide (NO) synthesis in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages was reduced by nigerin and ochracene J with their IC_{50} worths of 8.5 and 4.6 µM, respectively. Both compounds were recorded the potential antioxidants (Shang et al., 2021).

2.5. Cytotoxic Metabolites

In 1997, a diketopiperazine dimer, asperazine was isolated from the saltwater culture of *Aspergillus niger* which was acquired from *Hyrtios proteus* sponge taken from The Dry Tortugas National Park, Florida. The disc zone size of asperazine was evaluated against the standard chemotherapeutic agent cytosine arabinoside with the Corbett-Valeriote soft agar disc diffusion method. The first trial values µg/disc(mm): L1210/C38/H116 or CX1 were 50:400/40/300 for asperazine and 2.5:910/610/250 for cytosine arabinoside, respectively, then the second trial values, µg/disk: L1210/CFU-GM were 50:450/50 for asperazine, 0.5: >1000/45 for cyto-

sine arabinoside, respectively. According to these results of *in vitro* assay, the specific cytotoxicity of the alkaloid asperazine on leukemia cells was reported (Varoglu et al., 1997).

Bicoumanigrin A, aspernigrin A, aspernigrin B were isolated from *Aspergillus niger* (CBX-146-2002 strain) was acquired from the sponge *Axinella damicornis* Esper gathered from Scoglio della Triglia, south of Elba, Italy. Their antiproliferative effects were investigated against the human leukemia and carcinoma cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Bicoumanigrin A was showed a moderate cytotoxic effect with 8% (1 µg/mL), 48% (20 µg/mL) values, respectively. Aspernigrin A and aspernigrin B were considered very moderately cytotoxic by values of ranging from 5% to 15% at the maximum test concentration (50 µg/mL) (Hiort et al., 2004; Hiort et al., 2005).

α-pyrone derivatives, nigerapyrone B, nigerapyrone D, nigerapyrone E, asnipyrone A were isolated from the *Aspergillus niger* (MA-132 strain) was acquired from the internal tissue of *Avicennia marina* sea mangrove tree. Their cytotoxicity effects were examined on MCF-7, A549, Du145, HepG2, MDA-MB-231, HeLa, SW1990, NCI-H460 cell lines against positive control fluorourasil. IC_{50} values of fluorouracil were 52 µM (A549), 109 µM (HepG2), 3.3 µM (Du145), 31 µM (MCF-7), 121 µM (SW1990), 8.5 µM (NCI-H460), 59 µM (MDA-MB-231), none (HeLa). Nigerapyrone B was characterized as selective toxicity against the HepG2 cell line with an IC_{50} worth of 62 µM. Nigerapyrone D displayed weak or moderate cytotoxic with IC_{50} worths of 121, 81 and 81 µM on MCF-7, HepG2 and A549 cell lines, respectively. Nigerapyrone E was potent cytotoxic with IC_{50} worths of 43, 48 and 38 µM on A549, MDA-MB-231 and SW1990 cell lines, respectively; also was weakly or moderately cytotoxic with IC_{50} worths of 86, 86, 105 and 43 µM on HepG2, Du145, MCF-7 and NCI-H460 cells, respectively. Asnipyrone A is cytotoxic on the A549 cell line with an IC_{50} worth of 62 µM (Liu et al., 2011). In another report by the same team, nigerasterol A, nigerasterol B which represents the first marine-derived 5,9-epidioxysterol metabolites were isolated from *Aspergillus niger* (MA-132 strain) acquired from a fresh and healthy specimen of *Avicennia marina* sea mangrove plant. Their antiproliferative effects were investigated by SRB (sulforhodamine B) and MTT methods against HL60 and A549 cell lines with using positive control adriamycin. The IC_{50} values of adriamycin on cell lines HL60 and A549 were 0.11 and 0.43 µM, respectively. The IC_{50} values of nigerasterol A, nigerasterol B compounds were 0.30 and 1.50 µM on the HL60 cell line, 1.82 and 5.41 µM on the A549 cell line, respectively. Both compounds were reported to have strong antiproliferative effects (Liu et al., 2013).

Bis-naphto-γ-pyrone derivatives, aurasperone A, aurasperone B, aurasperone C, aurasperone F, asperpyrone A, asperpyrone D, asperpyrone E, fonsecinone A, fonsecinone B, fonsecinone C, fonsecinone D compounds were isolated from *Aspergillus niger* (SCSIO Jcsw6F30 strain) from brown algae *Sargassum* sp. All compounds were recorded have weak activity with the value of IC_{50} > 30 μM on the cytotoxicity test containing Du145, K562, MCF-7, A549, H1975, HL7702, HL60, Huh-7, Molt-4 and HeLa cell lines (Fang et al., 2016). A novel dimeric naphtopyrone derivative aurasperone H and a known polyketide derivative fonsecinone C were isolated from *Aspergillus niger* (2HL-M-8 strain) obtained from a mud specimen taken in the tidal zone of the Huludao coast, Liaoning Province, China. Their antiproliferative activities were investigated against A549, HL-60 and MGC-803 cell lines by MTT method with using paclitaxel as a positive control. Compared to paclitaxel used at a concentration of 10 mg/mL, aurasperone H exhibited a moderate inhibitor efficiency on cell lines A549 and HL-60, with IC_{50} worths of 67.1 and 11.5 μM, respectively, while fonsecinone C displayed remarkable antiproliferative effect on A549, HL-60 and MGC-803 cell lines, with IC_{50} worths of 2.8, 0.8 and 2.2 μM, respectively (Li et al., 2016).

A new ester furan derivative compound (unnamed) was isolated from *Aspergillus niger* (BRF-074 strain) acquired from marine sediment taken from the Northeast coastline of Brazil. The compound showed cytotoxic efficiency against HCT-116 cell line with an IC_{50} worth of 2.9 μg/mL (Uchoa et al., 2017).

A culture broth extract of *Aspergillus niger* (15F41-1-3 strain), obtained from an unknown sponge gathered on North Pagai Island, Indonesia, was not chose as a candidate to isolate bioactive substances including cytotoxic and antimicrobial agents. Afterwards, an interesting study was carried out by co-culture with *Mycobacterium smegmatis* mc^2 155 bacteria. *A. niger* culture extract underwent axenic conditions demonstrated growth inhibition rates of 2% and 48% against human prostate cancer DU145 cells at concentrations of 30 g/ml and 100 g/ml, respectively. Besides, *A. niger* culture extract co-cultured with *M. smegmatis* bacteria demonstrated growth inhibition rates of 55% and 96% at concentrations of 30 g/ml and 100 g/ml. As based on this development, cytotoxic compounds against DU145 cells were isolated under co-culture conditions. The isolated a pigment malformin C is active on the Du145 cell line with an IC_{50} worth of 0.11 μM. The creation of malformin C was shown to be the responsible for the increase in cytotoxic activity, while TMC-256A1, desmethylkotanin, and aurasperon C were specifically produced under co-culture conditions. For the first time, it has been documented that co-culturing a marine-derived *Aspergillus niger* (15F41-1-3) fungus

with *M. smegmatis* bacteria alters fungal secondary metabolite production (Jomori et al., 2020).

Fonsecin B, TMC-256A1, cyclo-(Leu-Ala), pentacyclic polyketide RF-3192C, dimeric coumarin orlandin, and cerebroside A were isolated from *Aspergillus niger* (ASSB4 strain). Cytotoxicity activities of all compounds were investigated against HepG2, HeLa, MCF-7, PC3 and A549 cancer cell lines with using positive control doxorubicin. Doxorubicin indicated EC_{50} values of 4.28, 1.45, 17.44, 8.64 μg/mL against HepG2, HeLa, MCF-7, A549 cell lines, respectively. Against HepG2 cell lines, orlandin, fonsecin B, TMC-256A1 and cerebroside A compounds gave the EC_{50} values of 45, 29.5, 30 and 20.8 μg/mL, respectively, while against MCF-7, A549 cell lines, RF-3192C compound gave the EC_{50} values of 47, 48.7 μg/mL, respectively. Each of fonsecin B and TMC-256A1 were moderately cytotoxic for the HepG2 cell line and compound RF-3192C was moderate-low cytotoxic for the MCF-7, A549 cell lines (Manar et al., 2021).

2.6. Enzyme and Protein Inhibitor Metabolites

Nafuredin, an epoxy-δ-lactone structure with a methylated olefinic side chain, was attained from *Aspergillus niger* (FT-0554 strain) was acquired from a sponge taken from Palau Republic, Palau Islands. In the *in vitro* study, the enzyme inhibition effect of nafuredin was evaluated on the several electron transport activities are NADH-fumarate reductase (complex I-II), NADH-ubiquinone reductase (complex I), NADH-rodoquinone reductase (complex I), rhodoquinol-fumarate reductase (complex II), succinate-ubiquinone reductase (complex II) systems in *Ascaris suum* (pig roundworm) mitochondria. The IC_{50} (nM) values of narufedin on adult *A. suum* were 12, 8, 24, 80,000 and >100,000, respectively. The IC_{50} (nM) values of nafuredin on NADH-ubiquinone reductase (complex I), NADH-rodoquinone reductase (complex I) systems of *A. suum* larval form were 8.9, 9.0, respectively. In the *in vivo* study, a sheep orally infected with 5000 *Haemonchus contortus* (wireworm) larvae and two control sheeps were treated orally with a 1% solution of nafuredin in an emulsifying solvent at 2 mg/kg. Fecal egg excretion, which was monitored for 11 days after the treatment, was observed to decrease by more than 90% on the 11th day. The IC_{50} (nM) values of nafuredin on NADH-ubiquinone reductase (complex I) and NADH-rodoquinone reductase (complex I) of *H. contortus* were 86 and 195, respectively. Nafuredin is thought to be a superior anthelmintic due to it is a very specific inhibitor of helminth complex I (Ōmura et al., 2001; Takano et al., 2001; Ui et al., 2001).

COX-2 is the inducible form of cyclooxygenase which is the pharmacologic target of nonsteroidal anti-inflammatory drugs (NSAIDs). Inhibi-

tion of COX-2 is essential in the discovery of new types of compounds with anti-inflammatory and analgesic effects. Accordingly, isolated compounds asperpyrone A, aurasperone C and aurasperone F from *Aspergillus niger* (SCSIO Jcsw6F30 strain) were evaluated for their COX-2 inhibition effects against positive control celecoxib. While celecoxib gave the IC_{50} worth of 0.011 μM, these substances exhibited significant activity with IC_{50} worths of 6.4, 4.2 and 11.1 μM, respectively (Fang et al., 2016).

A lack of the neurotransmitter acetylcholine, particularly in certain brain cell synapses, causes the progressive, multifactorial disease known as Alzheimer's. One of the most important factors causing this neurotransmitter deficiency is the acetylcholinesterase (AChE) enzyme activity, which hydrolyzes the acetylcholine released in the synaptic space. Thus, one of the ways to prevent this disease is the inhibition of this enzyme. Compounds of naphtopyrones derivatives rubrofusarin B, aurasperone A, asperpyrone B, asperpyrone C, flavasperone, fonsecinone A, and an alkaloid aspernigrin A were attained from *Aspergillus niger* acquired from a tunicate of *Phallusia nigra* collected from a coral reef in Hurghada, Egypt, Red Sea. In addition, silver nanoparticle (AgNP) forms of aurasperone A, aspernigrin A, and fonsecinone A compounds were synthesized. AChE enzyme inhibitory effects of all compounds were tested by the Ellman method with using galantamine as a positive control. Galanthamine showed an IC_{50} worth of 1.43 ± 0.36 μM, while rubrofusarin B compound showed a moderately inhibitor efficiency with an IC_{50} value of 13.87 ± 2.16 μM. Aurasperone A, fonsecinone A, and aspernigrin A compounds showed IC_{50} values of 4.90 ± 2.16, 7.52 ± 2.16, and 20.17 ± 3.03 μM, respectively, while silver nanoparticle forms which are aurasperone A AgNPs, fonsecinone A AgNPs, and aspernigrin A AgNPs showed stronger inhibitory effect with IC_{50} values of 0.311 ± 0.018, 0.089 ± 0.005 and 1.53 ± 0.076 μM, respectively (Abdelwahab et al., 2021).

Cholesterol which is produced sufficiently in the human body, is included in the structure of the cell membrane, has a remarkable role in the production of some hormones and in the protection of nerves. In particular, it has important effects on heart health. When cholesterol is good for the cardiovascular system, it is divided into high-density lipoprotein (HDL), and when it is bad, it is divided into low-density lipoprotein (LDL). Excess cholesterol in the bloodstream forms plaque in the arteries, leds to damage the flexibility of the vessels, hence blood flow slows down or is prevented and finally causes many diseases such as atherosclerosis, angina pectoris, heart attack (Ma and Shieh, 2006). Niemann-Pick C1-like 1 (NPC1L1) is a protein involved in the consumption of dietary cholesterol on the intestinal epithelium's exterior. As a result of the reactions occurring in the liver with the decrease of intestinal absorption of

cholesterol, LDL cholesterol in the bloodstream decreases, thus cardiovascular problems can be prevented. For this purpose, the discovery of compounds that inhibit the NPC1L1 protein has gained importance. Ezetimibe is the only drug that inhibits the transport activity of NPC1L1 (Long et al., 2021). In this regard, the isolated and semisynthetic compounds from *A. niger* (S-48 strain) which is obtained *K.candel* (L.) mangrove root is collected Beibu Bay of Guangxi Province, China were investigated. While naphto-γ-pyrones are fonsecinone A, fonsecinone C, aurasperone A, aurasperone E, asperpyrone B, asperpyrone C, rubasperone B, flavasperone, and coumarins are orlandin, 4,7-dimethoxy-5-methylcoumarin, 7-hydroxy-4-methoxy-5-methylcoumarin, desmethylkotanin were isolated directly from the fungus, by modifying the compounds fonsecinone A and aurasperone A, two new compounds comprised pyrazole ring were obtained by semisynthesis. The NPC1L1 inhibitor effects of all compounds were tested to reduce intestinal absorption of cholesterol against the positive control ezetimibe. Asperpyrone C among all compounds showed the highest inhibition activity with %47.8 against ezetimibe %56.6 at 100 μM concentration (Wu et al., 2022).

2.7. Insecticidal Metabolites

Pyranonigrin A (pyrano[3,2-b] pyrrole derivative) was attained from *Aspergillus niger* (CBX-146-2002 strain) which is associated with sponge *Axinella damicornis* Esper. Pyranonigrin A (100 ppm) led to that the average weight increase of the neonatal larvae of the plant insect *Spodoptera littoralis* over 7 days was reduced to 29%, while the survival rate was 85% (Hiort et al., 2004).

A new piperazinedione variant nigerpiperazine A and known fonsecinone A were attained from *Aspergillus niger* (JX-5 strain) acquired from *Ceriops tagal* mangrove inhabited in Dongzhaigang, Hainan county, China. The insecticidal effects of all compounds against *Helicoverpa armigera* Hubner were tested by serial dilution method using azadirachtin as a positive control. Compared to the IC_{50} worth of 50 μg/mL of azadirachtin, nigerpiperazine A and fonsecinone A compounds exhibited weak insecticidal efficiency with IC_{50} worths of 200 and 100 μg/mL, respectively (Bai et al., 2020).

2.8. Neuroprotective Metabolites

Aspernigrin B (6-pyridinone derivative) was isolated from *Aspergillus niger* (CBX-146-2002 strain) was acquired from the sponge *Axinella damicornis* Esper. The effect of Aspernigrin B on neuronal cells' intracellular calcium ($[Ca^{2+}]_i$) level is established. First, intracellular Ca was increased in neurons incubated with 200 μM L-Glutamic acid (L-Glu)

and 2.5 mM $CaCl_2$. Afterward, the $[Ca^{2+}]_i$ content in neurons treated with 1 µg/mL and 5 µg/mL Aspernigrin B showed values of 25.5% and 55.0%, respectively. Secondly, the $[Ca^{2+}]_i$ was increased in neurons incubated with 317 µM quisqualic acid (QUIS) and 2.5 mM $CaCl_2$. Afterward, the calcium content in neurons treated with 1 µg/mL and 5 µg/mL aspernigrin B showed worths of 33.6% and 23.5%, respectively. According to these results, Aspernigrin B can be a possible treatment option for neurodegenerative illnesses (Hiort et al., 2004).

Table 1. *Bioactive substances of marine-derived Aspergillus niger*

Compound	Fungal Strain	Marine Source	Chemical Structure	Activity	Reference
5,7-dihydroxy-2-[1-(4-methoxy-6-oxo-6H-pyran-2-yl)-2-phenylethylamino]-[1,4] naphthoquinone	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphthoquinoneimine	Moderate antifungal	Zhang et al., 2007b
6,9-dibromoflavasperone	MSA773	Sea mudflat	Brominated naphthopyranone	Strong antioxidant	Leutou et al., 2016
Asnipyrone A	MA-132	*Avicennia marina* (Mangrove)	α-pyrone	Cytotoxic (A549)	Liu et al., 2011
Asperamide A	EN-13	*Colpomenia sinuosa* (Brown algae)	Sphingolipid	Moderate antifungal	Zhang et al., 2007c
Asperazine	Ungiven	*Hyrtios proteus* (Sponge)	Alkaloid	Anti-leukemia	Varoglu et al., 1997
Asperitaconic acid A	LS11	*Haliclona* sp. (Sponge)	Itaconic acid derivative	Moderate antibacterial	Ding et al., 2018
Asperitaconic acid B	LS11	*Haliclona* sp. (Sponge)	Itaconic acid derivative	Weak antibacterial	Ding et al., 2018
Asperitaconic acid C	LS11	*Haliclona* sp. (Sponge)	Itaconic acid derivative	Weak antibacterial	Ding et al., 2018
Aspernigrin A	CBX-146-2002	*Axinella damicornis* (Sponge)	6-pyridinone derivative	Moderate anti-leukemia and carcinoma	Hiort et al., 2004
	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	2-benzylpyridin-4-one	Weak antiviral (HIV)	Zhou et al., 2016
	Ungiven	*Phallusia nigra* (Tunicate)	Alkaloid	Moderate AChE inhibitor	Abdelwahab et al., 2021
Aspernigrin A AgNPs	Ungiven	*Phallusia nigra* (Tunicate)	Alkaloid	Strong AChE inhibitor	Abdelwahab et al., 2021

Aspernigrin B	CBX-146-2002	*Axinella damicornis* (Sponge)	6-pyridinone derivative	Moderate anti-leukemia and carcinoma, Neuroprotective	Hiort et al., 2004
Aspernigrin C	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	2-benzylpyridin-4-one	High antiviral (HIV)	Zhou et al., 2016
Asperpyrone A	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic, Anti-inflammatory	Fang et al., 2016
Asperpyrone C	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphto-γ-pyrone	Weak antifungal	Zhang et al., 2007a
	S-48	*Kandelia candel* (Mangrove)	Naphto-γ-pyrone	NPC1L1 inhibitor	Wu et al., 2022
Asperpyrone D	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
Asperpyrone E	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
Aurasperone A	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	Ungiven	*Phallusia nigra* (Tunicate)	Naphtopyrone	Moderate AChE inhibitor	Abdelwahab et al., 2021
Aurasperone A AgNPs	Ungiven	*Phallusia nigra* (Tunicate)	Naphtopyrone	Strong AChE inhibitör	Abdelwahab et al., 2021
Aurasperone B	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphtho-γ-pyrone	Weak antifungal and Moderate antioxidant	Zhang et al., 2007a
	MSA773	Sea mudflat	Naphtho-γ-pyranone dimer	Strong antioxidant	Leutou et al., 2016
Aurasperone C	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic Anti-inflammatory	Fang et al., 2016
Aurasperone F	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic Anti-inflammatory	Fang et al., 2016
Aurasperone H	2HL-M-8	Mud sample	Naphthopyrone dimer	Moderate cytotoxic	Li et al., 2016
Averufin	MF-16	Sea water sample	Aflatoxin	Moderate Antiviral (TMV)	Wu et al., 2008

Bicoumanigrin A	CBX-146-2002	*Axinella damicornis* (Sponge)	3,3'-bicoumarin	Moderate anti-leukemia and anti-carcinoma	Hiort et al., 2004
Ester furan derivative compound	BRF-074	Sediment	Ester furan	Cytotoxic (HCT-116)	Uchoa et al., 2017
Flavasperone	MSA773	Sea mudflat	Naphtho-γ-pyranone monomer	Strong antioxidant	Leutou et al., 2016
Fonsecin	MSA773	Sea mudflat	Naphtho-γ-pyranone monomer	Strong antioxidant	Leutou et al., 2016
Fonsecin B	ASSB4	*Laurencia obtuse* (Red algae)	Naphtho-γ-pyrone	Moderate cytotoxic (HepG2)	Manar et al., 2021
Fonsecinone A	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	L14	*Reniera japonica* (Sponge)	Naphtho-γ-pyrone	Significant antibacterial	Liu et al.,2021
	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphtho-γ-pyrone	Weak antifungal	Zhang et al., 2007a
	JX-5	*Ceriops tagal* (mangrove)	Naphtho-γ-pyrone	Weak insecticide	Bai et al., 2020
	Ungiven	*Phallusia nigra* (Tunicate)	Naphtopyrone	Moderate AChE inhibitor	Abdelwahab et al., 2021
Fonsecinone A AgNPs	Ungiven	*Phallusia nigra* (Tunicate)	Naphtopyrone	Strong AChE inhibitor	Abdelwahab et al., 2021
Fonsecinone B	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphtho-γ-pyrone	Weak antioxidant	Zhang et al., 2007a
Fonsecinone C	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	2HL-M-8	Mud sample	Polyketide derivative	Antiproliferative	Li et al., 2016

Fonsecinone D	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Bis-naphto-γ-pyrone	Weak cytotoxic	Fang et al., 2016
	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphtho-γ-pyrone	Weak antioxidant	Zhang et al., 2007a
Germicidin C	LS24	*Haliclona* sp. (Sponge)	4-hydroxy-α-pyrone	Moderate antibacterial	Ding et al., 2019
Isoaurasperone A	L14	*Reniera japonica* (Sponge)	Naphtho-γ-pyrone	Significant antibacterial	Liu et al., 2021
Malformin A_1	MA-132	*Avicennia marina* (Mangrove)	Cyclopentapeptide	Weak antibacterial	Liu et al., 2013
Malformin C	15F41-1-3	Unidentified Sponge	Pigment	Cytotoxic (Du145)	Jomori et al., 2020
	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Cyclopentapeptide	Strong antiviral (HIV)	Zhou et al., 2016
	MA-132	*Avicennia marina* (Mangrove)	Cyclopentapeptide	Weak antibacterial	Liu et al., 2013
Nafuredin	FT-0554	Unidentified Sponge	Epoxy-δ-lactone	Antihelmintic	Omura et al., 2001, Ui et al., 2001, Takano et al., 2001
Nidurufin	MF-16	Sea water sample	Aflatoxin	Moderate Antiviral (TMV)	Wu et al., 2008
Nigerasperone C	EN-13	*Colpomenia sinuosa* (Brown algae)	Naphtho-γ-pyrone	Weak antifungal Moderate antioxidant	Zhang et al., 2007a
Nigerasterol A	MA-132	*Avicennia marina* (Mangrove)	Sterol	Antiproliferative (HL60, A549)	Liu et al., 2013
Nigerasterol B	MA-132	*Avicennia marina* (Mangrove)	Sterol	Antiproliferative (HL60, A549)	Liu et al., 2013
Nigerin	164117	*Dysidea* sp. (Sponge)	Sesquiterpenoid	Strong antioxidant	Shang et al., 2021
Nigeropyrone B	MA-132	*Avicennia marina* (Mangrove)	α-pyrone	Selective cytotoxic (HepG2)	Liu et al., 2011
Nigeropyrone D	MA-132	*Avicennia marina* (Mangrove)	α-pyrone	Weak-Moderate cytotoxic (MCF-7, HepG2)	Liu et al., 2011
Nigeropyrone E	MA-132	*Avicennia marina* (Mangrove)	α-pyrone	Strong citotoxic (A549, SW1990, MDA-MB-231)	Liu et al., 2011
Nigerpiperazine A	JX-5	*Ceriops tagal* (mangrove)	Piperazinedione derivative	Weak insecticide	Bai et al., 2020

Nipyrone A	LS24	*Haliclona* sp. (Sponge)	4-hydroxy-α-pyrone	Moderate antibacterial	Ding et al., 2019
Nipyrone B	LS24	*Haliclona* sp. (Sponge)	4-hydroxy-α-pyrone	Moderate antibacterial	Ding et al., 2019
Nipyrone C	LS24	*Haliclona* sp. (Sponge)	4-hydroxy-α-pyrone	Strong antibacterial, Moderate antituberculosis	Ding et al., 2019
Ochracene J	164117	*Dysidea* sp. (Sponge)	Sesquiterpenoid	Strong antioxidant	Shang et al., 2021
Orlandin	ASSB4	*Laurencia obtuse* (Red algae)	Dimeric coumarin	Weak cytotoxic (HepG2), High antibacterial	Manar et al., 2021
Pyranonigrin A	CBX-146-2002	*Axinella damicornis* (Sponge)	Pyrano[3,2-b]pyrrole derivative	Insecticide	Hiort et al., 2004
RF-3192C	ASSB4	*Laurencia obtuse* (Red algae)	Pentacyclic polyketide	Moderate-low cytotoxic (MCF-7, A549), High antibacterial	Manar et al., 2021
Rubrofusarin	SCSIO Jcsw6F30	*Sargassum* sp. (Brown algae)	Naphtopyrone	Weak antiviral (HIV)	Zhou et al., 2016
TMC-256A1	ASSB4	*Laurencia obtuse* (Red algae)	Naphtopyrone	Moderate cytotoxic (HepG2)	Manar et al., 2021
	Ungiven	Ungiven	Ungiven	Antiviral (IRES)	Ovenden et al., 2004
	MSA773	Sea mudflat	Naphtho-γ-pyranone monomer	Strong antioxidant	Leutou et al., 2016
Yanuthone A	F97S11	*Aplidium* sp. (Ascidian)	Farnesylated epoxy cyclohexenone	Antibacterial	Bugni et al., 2000

3. CONCLUSION

Marine habitats are a new resource in the seek for novel medicines for the therapy of various diseases. Marine fungi are inhabited by algae, seagrass, marine vertebrates/invertebrates and also seawater, sediments, hydrothermal vents, mud, mudflat. Marine fungi adapt and resist to extreme sea conditions. In addition to marine creatures, fungi, which are in symbiotic relationships with these creatures, have exhibited new secondary metabolites thanks to their resistance to extreme environmental conditions. One of the marine-derived fungi, *Aspergillus niger* is an important pharmaceutical resource of new secondary metabolites which has activity such as antimicrobial, cytotoxic, antioxidant, neuroprotective and enzyme-protein inhibitions. In the discovery for novel antibiotics, anticancer medicines, and new types of drugs to be used in the therapy of Alzheimer's and nervous system diseases, it is recommended that studies on marine-derived *Aspergillus niger* fungus increase.

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Chapter 6

LARYNX ANATOMY, CLINICAL EFFECT OF OSSIFICATION IN LARYNX CARTILAGES

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The breathing process performed to meet the oxygen needs of our cells, tissues and organs and to expel the carbon dioxide formed is called respiration. The respiratory system, which is necessary for us to perform the respiratory function; consists of the airways and lungs. Respiratory tracts respectively; consists of the nose (nasus), pharynx, larynx, trachea, and bronchi. During the breathing (inspiration) process, the air entering through the nose follows the structures forming the airways and reaches the lungs. With the expiration process, carbon dioxide in the lungs passes through the bronchi, trachea, larynx, pharynx, respectively, and is thrown out through the mouth.

The respiratory tracts are divided into two parts, the upper respiratory tracts and the lower respiratory tracts. Upper respiratory tracts consist of the nose, pharynx, and larynx. The lower respiratory tracts consist of trachea, bronchi, and bronchioles. In some sources, the larynx is considered to be from the lower respiratory tract.

LARYNX

Larynx is the part of the respiratory tract that comes after the pharynx. It is an organ made of cartilage, muscle and membranes. It is located at the level of 3-6. cervical vertebrae in adult males and slightly higher in children and females. It is located at the level of 2-4. cervical vertebrae in newborns and infants (6-12 months). Since the larynx is attached to the hyoid bone via ligaments and muscles, it moves with the hyoid bone.

Functions of the larynx

- It transmits the air taken during respiration to the lower respiratory tract.
- It prevents saliva and nutrients from escaping into the lower respiratory tract during the swallowing process.
- It is where the sound (phonation) occurs. As the air trapped in the lungs passes through the larynx cavity, it vibrates the vocal cords in the plica vocalis and phonation occurs.

Laryngeal cavity (Cavitas larynges)

The space inside the larynx is called cavitas laryngis. The aditus larynges (laryngeal inlet), called the larynx entrance, is the gateway connecting the larynx to the pharynx. Cavitas laryngis is divided into three parts from top to bottom.

- Vestibule of larynx (Vestibulum laryngis): It is the first part between aditus laryngis and plica vestibularis.

- Ventricle of larynx (Ventriculus laryngis): It is the small section in the form of a pocket between the plica vestibularis and the plica vocalis.
- Infraglottic cavity (Cavitas infraglottica): It is the part below the plica vocalis and extends to the trachea.

CARTILAGINES OF LARYNX

The larynx is made up of cartilages. There are 9 cartilages in the larynx, 3 single and 3 double. While some of the larynx cartilages begin to ossify over time, some do not ossify.

Thyroid cartilage (Cartilago thyroidea): Thyroid cartilage is the largest cartilage of the larynx. It consists of two separate laminae, the lamina dextra on the right and the lamina sinistra on the left. Angulus thyroidea is the angle formed by the laminae joining anteriorly and posteriorly. This angle is about 90º for men and about 120º for women. The large protrusion in the anterior part of the cartilage is called prominentia laryngea (Adam's apple). While this protrusion is prominent in adult males, it is less prominent in females and children. Prominentia laryngea protrudes more anteriorly in males, causing the plica vocalis to be longer. That's why adult men's voices are deeper than women's and children's voices. Between the thyroidea cartilage and the hyoid bone is the membrana thyrohyoidea. Thanks to this ligament, when the hyoid bone moves, the thyroid cartilage also moves.

Cricoid cartilage (Cartilago cricoidea): It is the thickest and strongest cartilage among the larynx cartilages. From the outside, it looks like a stone ring. In front of the cartilage, there is a narrow arch called arcus cartilaginis cricoidea. On the back, it has a wide lamina named lamina cartilaginis cricoidea. At the bottom, it is connected to the first cartilage of the trachea via the ligamentum cricotracheale. Above, it attaches to the thyroidea cartilage by the ligamentum cricothyroideum.

Epiglottic cartilage (Cartilago epiglottica): It is located anterior to the vestibule of larynx and is leaf-shaped. It is in the structure of elastic cartilage and does not ossify over time. Vascular and nerve passages occur through the holes on the anterior and posterior surfaces of the epiglottic cartilage. There is a pedicle called petiolus epiglottidis and this pedicle is attached to the thyroid cartilage via the thyroepiglottic ligament. It provides the closure of the entrance part of the epiglottis larynx (aditus laryngis) during swallowing. In this way, the escape of nutrients to the trachea and lungs is prevented.

Arytenoid cartilage (Cartilago arytenoidea): They are cartilages in pairs located on the posterior side of the larynx and on the lateral sides of the upper edge of the cartilago cricoidea. It resembles a pyramid in appearance. The apex articulates with the corniculate cartilage. There is a processus vocalis protrusion on the front of the base and attaches here to the ligamentum vocale (vocal cords). Processus vocalis part is in the structure of elastic cartilage and ossification is not observed. On the back of the base is the processus muscularis.

Corniculate cartilage (Cartilago corniculata=Santorini): Two small cartilages in the form of cones or horns located at the top of the arytenoid cartilage.

Cuneiforme cartilage (Cartilago cuneiformis=Wrisberg): It is the cartilages located in front of the corniculate cartilage in the aryepiglottic fold.

LARYNX'S JOINTS AND LIGAMENTS

The joints connecting the cartilages of the larynx are movable joints and these are articulatio cricothyroidea and articulatio cricoarytenoidea.

Thyrohyoid membrane (Membrane thyrohyoideum): The thyrohyoid membrane, located between the upper border of the thyroid cartilage, the greater horn and the body of the hyoid bone, consists of elastic fibrous connective tissue. The thick part of the thyrohyoid membrane, located between the tip of the greater horn of the hyoid bone and the superior horn of the thyroid cartilage, is called the lateral thyrohyoid ligament. The section between the posterior surfaces of the hyoid bone and the superior thyroid notch is called the medial thyrohyoid ligament. The structures that pass through the thyrohyoid membrane are the ramus internus of the superior laryngeal artery, the superior laryngeal vein, and the superior laryngeal nerve.

Cricothyroid joint (Articulatio cricothyroidea): It is a joint formed between the medial aspect of the inferior horn of the thyroid cartilage and the anterolateral aspect of the cricoid cartilage. There is a capsule surrounding the joint, and the ceratocricoid ligament strengthens the capsule.

Cricoarytenoid joint (Articulatio cricoarytenoidea): The joint formed between the articular surface at the upper edge of the lamina cartilaginis cricoidea and the articular surface at the base of the arytenoid cartilage is called the cricoarytenoid joint. The capsule surrounding the joint is called the capsula cricoarytenoidea, and the ligament that strengthens the joint is called the cricoarytenoid ligament. It connects with the petiolus epiglottidis thyroepiglottic ligament to the medial aspect of the

angulus thyroideus. The ligament extending between the epiglottis and the upper edge of the hyoid bone is called the hyoepiglottic ligament.

Fibroelastic membrane of larynx (Membrana fibroelastica laryngis): This membrane, located in the lower part of the mucosa of the larynx, provides the protection of the larynx in shape. Some parts thicken to form ligaments. Membrane fibroelastica laryngis consists of two parts, membrana quadrangularis and conus elasticus (membrana cricovocalis).

Quadriangular membrane (Membrana quadriangularis): Quadriangular mambrane, which is the part of the membrane fibroelastica laryngis above the ventriculus laryngis, attaches anteriorly to epiglottic cartilage, posteriorly to arytenoid cartilage and corniculate cartilage. It forms the plica aryepiglottica with its upper edge, and the ligamentum vestibulare (ventriculare) at its lower edge, which attaches to the angulus thyroideus anteriorly and to the arytenoid cartilage posteriorly. The membrane quadriangularis is covered by the mucosa and forms the plica vestibularis. The space between the plica vestibularis is called the rima vestibuli.

Conus elasticus (Membrana cricovocalis): The portion of the membrane fibroelastica laryngis inferior to the ventriculus laryngis is known as the conus elasticus. The thick part of the conus elasticus in the anterior is called the ligamentum cricothyroideum medianum, and the thinner part in the lateral is called the pars lateralis.

The thicker part of the conus elasticus between the angulus thyroideus and the processus vocalis is called the ligamentum vocale. The space between the plica vocalis, which is longer than the plica vestibularis, is called the rima glottidis.

INTRINSIC MUSCLES OF LARYNX

Larynx muscles are examined in two groups as external muscles and internal muscles. The muscles that are between the larynx and the neighboring formations and enable the larynx to move during swallowing are called the "external muscles of the larynx".

The muscles that lie between the cartilages of the larynx itself and are involved in the movement of the vocal cords and the opening and closing of the larynx entrance are called the "internal muscles of the larynx".

1. Cricothyroid muscle (Musculus cricothyroideus=Musculus anticus): It stretches the vocal cords and closes the rima glottis.

2. Lateral cricoarytenoid muscle (Musculus cricoarytenoideus lateralis): Adducts the vocal cords and closes the anterior part of the rima glottis.

3. Posterior cricoarytenoid muscle (Musculus cricoarytenoideus posterior = Musculus posticus): Abducts the vocal cords and opens the rima glottis.

4. Musculus vocalis: It changes the vibration of the vocal cords.

5. Transverse arytenoid muscle (Musculus arytenoideus transversus): It closes the posterior part of the rima glottis.

6. Oblique arytenoid muscle (Musculus artenoideus obliquus): It pulls the arytenoids towards each other

7. Thyroarytenoid muscle (Musculus thyroarytenoideus): pulls the thyroid cartilage towards the arytenoid cartilage, thereby relaxing the vocal cords.

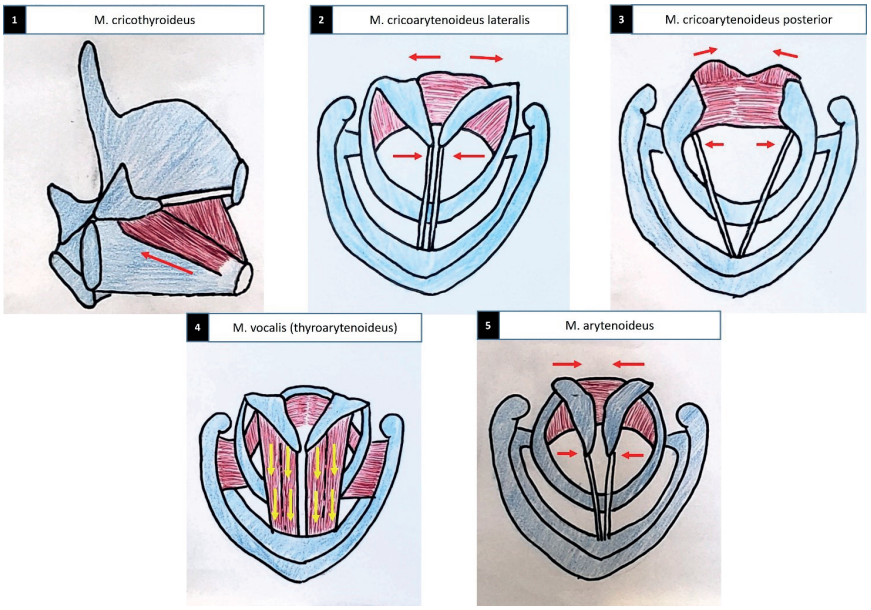

Figure 1. *Intrinsic muscles of larynx*

CLINICAL INFORMATION

Hoarseness occurs when the nervus laryngeus recurrens is cut unilaterally or damaged (especially during thyroid surgeries). If the nervus laryngeus recurrens is cut on both sides, breathing difficulties occur.

Foreign bodies escaping into the larynx (especially common in infants) may close the rima glottis. In this case, urgent intervention is required as the respiratory tract will be closed.

LARYNX VESSELS AND NERVES

Larynx Arteries

Arteria laryngea superior, which is the branch of the arteria thyroidea superior, and arteria laryngea inferior, which is the branch of the arteria thyroidea inferior

Larynx Veins

It collects the larynx venous blood "vena laryngea superior" and "vena laryngea inferior". "The vena laryngea superior opens to the vena jugularis interna via the vena thyroidea superior, and the vena laryngea inferior opens to the vena brachiocephelica sinistra through the vena thyroidea inferior.

Larynx Lymphatics

The lymphatics of the larynx are divided into two parts, superior and inferior to the plica vocalis. The superior lymphatic vessels drain into the deep lymph nodes of the neck, and a part of the inferior group drains into nodi prelaryngeales and nodi pretracheales. The other part of the inferior group drains into the deep lymph nodes of the neck and nodi supraclavicularis.

Larynx Nerves

They are innervated by branches of the nervus vagus, these are nervus laryngeus superior and nervus laryngeus recurrens (inferior). Nervus laryngeus superior has two branches, ramus internus and ramus externus. Of these branches, ramus internus contains sensory and autonomic fibers, and ramus externus contains sensory and motor fibers. The ramus internus pierces the membrana thyrohyoidea and forms the plica nervi laryngis inferior to the recessus piriformis, the sense of the area up to the plica vocalis belongs to the ramus internus. The ramus externus is only responsible for the innervation of the musculus cricothyroideus. Nervus laryngeus recurrens contains motor and sensory fibers. Nervus laryngeus recurrens innervates all the intrinsic muscles of the larynx except the musculus cricothyroideus. The larynx sense inferior to the plica vocalis also belongs to the nervus laryngeus recurrens.

OSSIFICATION

Calcium salts, which normally accumulate in the bone, can sometimes be deposited in soft tissue. Precipitation of calcium salts occurs mainly in the form of calcium phosphate. The unorganized accumulation of minerals in soft tissue is called 'heterotopic calcification', while the

cases where they accumulate in an organized manner are known as 'heterotopic ossification'.

Soft tissue calcifications/ossifications are frequently observed in individuals over the age of 40, but there are also a few studies showing that they are observed in children. Calcifications or ossifications do not cause any significant signs or symptoms. It is usually detected incidentally during radiographic examination. The prevalence of soft tissue calcifications has been reported to vary between 2% and 5%.

When soft tissue calcifications are detected, the primary goal is to recognize the calcification and determine if treatment is needed. For a correct diagnosis, criteria such as localization, number, shape and distribution of calcification should be considered. In addition, it is important to know the soft tissue anatomy well. Differential diagnosis of these calcifications should be made on radiographs with anatomical structures such as hyoid bone, triticeous cartilage, styloid process, epiglottis and upper horns of thyroid cartilage. When soft tissue calcifications are adjacent to bone, it is difficult to determine whether the calcification is within the bone or in the soft tissue. In such cases, it may be useful to detail the patient's history and clinical examination, to take a radiograph from a different angle, or to resort to advanced imaging techniques.

The structures with the most common calcification tendency in the head and neck region are laryngeal cartilages, vertebrae, arteries and thyroid gland.

Laryngeal cartilage calcifications and ossifications

Most of the laryngeal cartilages are composed of hyaline cartilage, while the epiglottis and arytenoid cartilages are composed of fibroelastic cartilage. Between the thyrohyoid ligaments is a small pair of triticeous cartilages. Both thyroid and triticeous cartilages contain hyaline cartilage, and hyaline cartilage has a tendency to ossify and calcify with age. Ossification of the laryngeal cartilages usually occurs asymmetrically, especially in the thyroid cartilage. Triticeous cartilages are bilateral ovoid structures located in the laryngeal skeleton. Although its functions are not known exactly, the opinion that has been put forward recently is that it strengthens the thyrohyoid ligament. The thyroid cartilage is the uppermost part of the larynx cartilages and is also the largest. Calcification of the thyroid cartilage continues throughout life. The calcification process, which usually starts from the posterior border, reaches the lower horn of the thyroid cartilage, and the calcification process is completed around the age of 70.

Figure 2. *Fully ossified thyroid cartilage.*

Clinical features

Laryngeal cartilage calcification is encountered incidentally during radiographic examination and has no clinical findings.

Radiographic features

The most common laryngeal cartilage calcification in panoramic radiographs; calcified triticeous and thyroid cartilages. Calcified triticeous cartilage is observed under the greater horn of the hyoid bone and adjacent to the upper border of the 4th cervical vertebra on panoramic and lateral head radiographs. Triticeous cartilage varies between 7-9 mm in length and 2-4 mm in width. Calcified triticeous cartilage shows a prominent and smooth structure. The superior horn of the calcified thyroid cartilage is located medial to the 4th cervical vertebra and is superposed to the prevertebral soft tissue. Calcified laryngeal cartilage usually shows a homogeneous radiopacity, but sometimes an outer cortex can be observed. Radiologic findings of laryngeal cartilage calcifications are not frequently observed in children and infants under 13 years of age.

Differential diagnosis

Calcified triticeous cartilage may be confused with calcified atherosclerotic plaque in the carotid bifurcation. However, its solitary nature and homogeneous structure are important in the differential diagnosis. It is extremely important to distinguish calcified atherosclerotic plaques, which pose a risk for cardiovascular and cerebrovascular diseases, from calcified triticeous cartilages. There is no treatment requirement for laryngeal cartilage calcifications.

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Chapter 7

VERMIFORM APPENDIX: THE ANATOMICAL VARIANTS AND CLINICAL VIEW

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Introduction

The vermiform appendix, a small, blind-ended tubular sac, is a true diverticulum, and is located roughly 1.7 cm beneath the ileocecal valve arising from the posteromedial cecal margin (1-3). According to Terminology Anatomica, its name is "appendix vermiformis" in Latin (2, 4). The term "vermiform" means "worm-like" and refers to the thin, tubular structure. Berengario da Carpi first defined the vermiform appendix in 1521. Claudius Amyand performed the first appendectomy in 1736. Amyand also described an inguinal hernia involving the appendix in the same surgical case (Amyand's Hernia). The vermiform appendix is in whole hominins, including monkeys and humans. Also, many other mammals, including rabbits, and wombats involved a cecal appendix (1).

It is located in front of the iliopsoas muscle and the lumbar plexus and behind the greater omentum and/or the anterior abdominal wall. The vermiform appendix is connected to the lower part of the mesenterium of the ileum by a short, triangular mesoappendix, which surrounds the appendicular artery and the inferior ileocecal recess. The mesoappendix runs throughout the whole length of the appendix (5). Because the mesoappendix is shorter than the appendix, the vermiform appendix has a curved view. Although the distal lumen is ordinarily partly clogged, the proximal end of the organ opens into the cecum approximately 2.5 cm beneath the valva ileocaecalis (Bauhin's valve). The fold of mucous membrane, called Gerlach's valve, protects this gateway (1).

The organ has an average length of about 9 cm. This length generally ranges from 1.5 cm to 25 cm (6-8). It is not an acquired diverticulum. As a true diverticulum, it includes whole layers of the colon such as the mucosa, submucosa, serosa, and longitudinal and circular muscularis propria (2). Tunica mucosa and tela submucosa are thicker and have abundant lymphoid structures on all sides. Longitudinal muscle fibers are evenly distributed throughout. Therefore, taenia seen in the large intestine are not found. Circular muscle fibers are more developed than longitudinal fibers (5). The histological difference between the colon and vermiform appendix is due to the presence of B and T lymphoid cells in the mucosa and submucosa layers of the appendix (2). Its physiological function has long been the issue of discussion, and the organ is often regarded as a remnant of evolutionary development. However, recent findings on comparative primate anatomy and immune function prove otherwise (1).

The neuroendocrine cells in the mucosa layer produce amines and hormones while the lymphoid tissue ensures the maturation of B lymphocytes and the production of IgA antibodies (2, 9). Researches into its function in humans is still ongoing. However, because of the gut-asso-

ciated lymphoid tissue (GALT) found in the lamina propria layer, its role in immunity has begun to be discussed. For the most part, the vermiform appendix was assumed to be a vestigial organ and has maintained its reputation in this regard (2). But recently, advances in scientific studies on gut immunity have demonstrated that the vermiform appendix is a "safe home" for symbiotic gut microbes (2, 10). While the substantial amount of lymph tissue inside is accepted to let the growth of diverse colonies of useful bacteria in the gut, its slim, elongated form can help prevent the loss of these colonies due to diarrhea (1, 10). In fact, it is not a primitive organ. It is a considerable part of the immune system that has a prominent function in GALT, unlike lymphoid tissue in other parts of the gut. Examining the evolutionary features of the organ, it can be deduced that originally its main function was to interact with and process intestinal bacteria. It stimulates the development of GALT, re-colonises the colon with commensal flora and contributes to the recovery process after diarrhea (11). This indicates an evolutionary supremacy for preserving the worm-shaped vermiform appendix and undermines the thesis that the organ was a primitive organ (2, 4, 11).

Embriyology

The cecum, appendix, the small intestine below the first half of the duodenum, the proximal two-thirds of the transverse colon, and the ascending colon develop from the embryonic midgut. In the embryonic period, the intestines are connected by the superior mesenteric artery, which forms the midgut loop, at 6 weeks of development. The cecum bud develops from the caudal piece of the loop (12). The growth of the cecal bud matches the growth of the colon. The lower part cannot keep up with this growth rate. When this difference grows, the lower part separates from the cecum, and forms the appendix. Thus, the length of the organ begins to increase speedily (1). The vermiform appendix emerges from the midgut. Cecal diverticulum, as the pioneer of the cecum and appendix, comes in sight in the 6th week (2). The vermiform appendix can be seen histologically at the 8th week of pregnancy (1, 2). As a result of elongation of the colon, the vermiform appendix and cecum turn medially with the midgut and descend towards the right lower abdomen. It has various random positions because it is pushed towards the anteriorly of the cecum (2, 13). The circular part of the lumen turns into a lobed structure in the 12th week (1). At 14th-15th weeks, the mucosa develops lymphoid tissue, which will contribute to immune function. Lymph nodes become clear and proceed to grow from postnatal to adolescence in the 4th and 5th months (1, 2, 12).

The Blood Supply, Innervation and Lymphatics of Appendix

Arterial supply is from the appendicular artery (Fig. 1). When the literature is examined, there is no consensus on the origin of the main appendicular artery. In a number of studies, it has been determined that it originates only from the ileocolic artery, while in others it has been found that it originates from one of the branches of the ileocolic artery. Noting the presence of accessory appendicular arteries, most authors argue that they always originate from the anterior or posterior cecal artery. However, in the study of Swathipriyadarshin et al., an artery originating from the anterior cecal artery was not observed. It was found that the main appendicular artery emerged mostly from the lower branch of the superior mesenteric artery (46.88%), then from the ileal branch (28.13%), ileocolic artery (18.75%) and arterial arcade (6.25%), respectively (14).

According to the results of another study, the main appendicular artery originated from the ileocolic artery (35%), the posterior cecal artery (13%), the anterior cecal artery (4.25%), the medial branch of the ileocolic artery (8.25%), and the ileum branch (4.25%). In the same study, it was found that 4.25% of the appendicular artery originated from an arcade between the ileocolic artery and its ileum branch, and 30.5% from the terminal ring of the ileocolic artery (15).

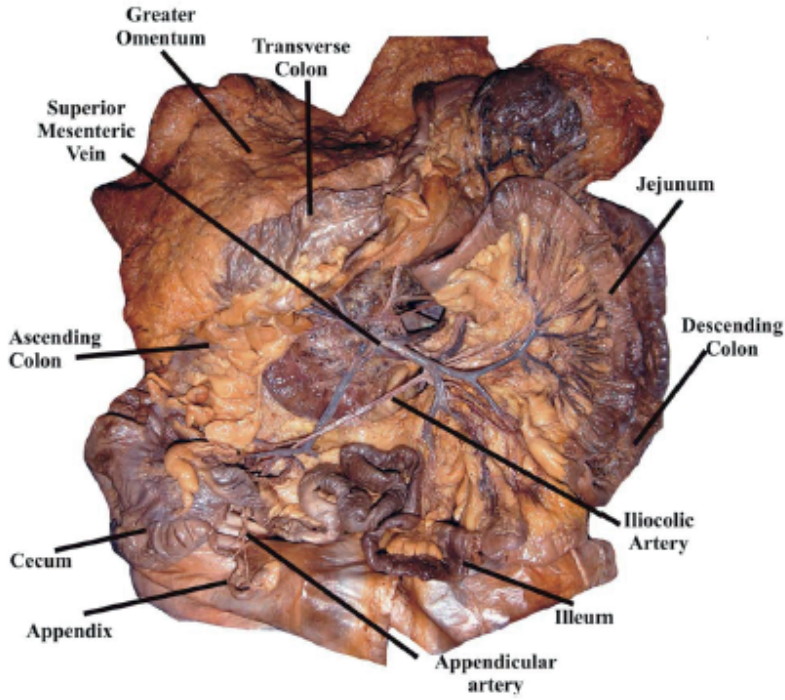

Fig. 1. *Appendicular Artery in a Cadaveric Dissection (1)*

The main artery, which provides blood supply to the appendix vermiformis, passes behind the last part of the ileum and enters the mesoappendix near its basis. It gives a returning branch in here. This returning branch anastomoses at the beginning of the vermiform appendix with a branch of the posterior caecal artery. These branches that make up the anastomosis can sometimes be very thick. The main artery of the appendix first comes close to the free edge of the mesoappendix, then follows its free edge to its apex. The last part of the artery runs within the vermiform appendix wall, so the lumen of the artery may be occluded in the inflammation of this organ. As a result, distal gangrene and necrosis may develop. The distribution scheme of this artery shows a great deal of variation. In addition, two or more accessory arteries may come in 80% of cases (5).

The appendicular vein located within the mesoappendix, drains into the ileocolic vein. Thereafter it drains into the superior mesenteric vein; therefore, infection in the appendix can be transported to the liver via the portal vein (1).

The parasympathetic innervation of the appendix is provided by the vagus nerve. Sympathetic innervation is provided from T10-L1 branches of the celiac ganglia and superior mesenteric plexus. Sensory nerves are connected to the 10th thoracic nerve, following the sympathetic nerves (16).

The lymphatic vessels of the cecum and vermiform appendix drain to the appendicular lymph nodes in the mesoappendix and to the ileocolic lymph nodes along the ileocolic artery; they then open into the superior mesenteric lymph nodes (5).

The Congenital Anomalies and Anatomical Variants

Developmental abnormalities of the organ are extremely infrequent (1, 17). It is thought that gender, age, race or other demographic parameters may affect the location of the appendix (8, 18, 19).

According to Vieira's study, the highest mean recorded for appendix length was 10.21 cm in male and 8.03 cm in female. Age was a parameter remarkably associated with appendix length. While it was longer in children and adolescents, it gradually decreased with advancing age. Therefore, age is a parameter that can be interrelated with a high rate of acute appendicitis in children and young individuals, and infrequently seen in individuals over fifty years of age. Since the appendix is longer in male than in female, it can be concluded that gender also affects the length of the appendix (20). The organ is typically located on the surface of the cecum and at the junction of its three taenia coli (17). In other words it

located in the lower right quadrant of the abdomen (1, 17). While the base of the vermiform appendix is fixed where it attaches to the cecum, its tip can be in diverse positions. The position variations of the appendix are divided into six categories: pelvic, retrocecal, subcecal, paraileal, retroileal, and subhepatic position (Fig. 2) (17, 21). Unlike these positions, the vermiform appendix can be located in the left upper quadrant, left anterior paramid line and lower midline.

In addition, the position of the organ is affected by changes in posture, breathing, abdominal muscles tone, and changes in the cecum, depending on the degree of stretching of the adjacent intestines (1, 12).

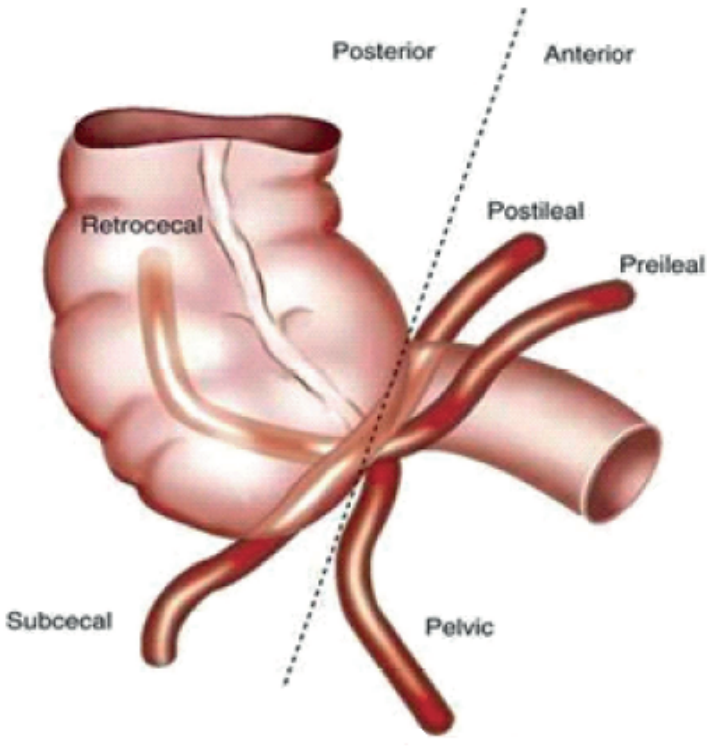

Fig. 2. *Anatomical Localization of The Appendix (17)*

In a research of 10,000 people, 65% retrocecal and retrocolic, 31% pelvic, 2.2% subcecal, 1% preileal, and 0.4% postileal appendix positions were determined. The vermiform appendix, located in the pelvic position, is closely adjacent to the right ovary and right tuba uterina in women. It is in connection with the anterior abdominal wall in the preileal position (5). Retrocecal position (43%) was the most common anatomic position in 75 cases of appendicitis diagnosed with CT. This was followed by the pelvic

(33%), subcecal (13%), post ileal (8%) and preileal (3%) positions. In the same study, the most common position in men was retrocecal (50%). This was followed by the subcecal position (18%). In women, the most common pelvic position (43%) was followed by the post-ileal position (11%). The incidence of pre-ileal vermiform appendix position (1%) was equal in both men and women patients (17). In some studies, the rate of the subcecal localization of the vermiform appendix was higher in men than in women (17, 22, 23).

Although the variability of the vermiform appendix is rarely seen, this variability usually manifests itself as agenesis, duplication and horse-shoe-shaped appendix (24, 25). In Mehmet Ali's case study, patient had no right piriformis on CT, while the left piriformis was normal. In addition, the vermiform appendix had passed through the greater sciatic foramen, and located in the right deep gluteal region (25). Fawcitt also presented a study showing that the vermiform appendix is located in the thorax due to intestinal malrotation and diaphragmatic defect (26). Lamture et al. suggested that the only organ that does not have a fixed anatomical position is the vermiform appendix (27).

In typical appendicitis, pain occurs clinically in the lower right quadrant of the abdomen. However, in the presence of a long appendix localized in a different location, symptoms may occur in different parts of the abdomen and may lead the clinically misdiagnosis (7, 8). To understand where pain occurs during appendicitis, it is necessary to know the variational positions of the appendix. In consequence of the irritation of the ureter, the retrocecal appendix may show symptoms of upper urinary tract infection. Pain may be felt in the vermiform appendix in the pelvic location, as the obturator internus muscle is stretched when the thigh is flexed and rotated medially. The vermiform appendix in the pelvic position can irritate the bladder or rectum. Therefore, it can cause symptoms such as pain when urinating, suprapubic pain, or feeling the need to defecate. In some male patients, the postileal vermiform appendix position may cause irritation of the ureter, which may lead to testicular pain in patients (17). During the physical inspection, major attention should be paid to the anatomical variative of the vermiform appendix. Because in some patients, such as the retrocecal appendix position, the diagnostic findings may be completely different from normal (28). Krüger's sign is a rather significant finding in physical inspection since it has a sensitivity of up to 90% in the retrocaecal appendix position (29). Determining the diagnosis in acute appendicitis may be delayed owing to the position of the vermiform appendix. Therefore, the retrocecal vermiform appendix position may be one of the factors that can cause peritonitis or inflammatory alterations in the retroperitoneal fascia and adipose tissue (28). Palpation findings

may sometimes be negative due to the specific position of the retrocaecal appendix and the diagnosis may be "silent" appendicitis, which is not the usual symptom of appendicitis, resulting in the development of many different complications (30). Variations in appendix length are also an important criterion for surgeons and radiologists. Diagnosis may be inadequate because the infected appendix tip does not reach the mean length. Aynı zamanda this delay may cause perforation and gangrene (17). Zacharzewska et al. presented extraordinary locations of the tip of the long vermiform appendix, or normal appendix in CT. One patient had left-sided mid-gut malrotation of the cecum and two patients had situs inversus. In these patients, the vermiform appendix was in the pelvic position and was located in the left lower quadrant of the abdomen (8, 31). In this situation, left-sided appendicitis may be mistakenly confused with acute diverticulitis (31). In a few cases with long retrocecal and retrocolic location or high localization of the cecum, the tip of the appendix was found to be located subhepaticly throughout the inferior border of the liver and/or close to the right kidney.

Appendicitis developing in the subhepatic position can be confused with the diagnosis of right renal colic, cholecystitis, liver abscess or pyelonephritis (8, 32-35). The tip of the long vermiform appendix can also be viewed near the part of the descendens duodenum or even the horizontal duodenum. Inflammation of the tip of the worm-shaped appendix located close to the duodenal wall can cause symptoms of duodenitis or duodenal ulcer. Sometimes long, pelvic vermiform appendix may cross the right ureter (8). A long vermiform appendix that crosses the right ureter may bring about hydronephrosis with symptoms of renal colic because of infection or abscess (36). The tip of the vermiform appendix can also be located in the midline below and anterior to the aortic bifurcation (8). Appendicitis can mimic enteritis when the tip of the appendix is in the midline (37). The long pelvic appendix can reach the rectum wall. In a long pelvic appendix location, there is also the possibility that the appendix is located in the scrotum as the contents of the inguinal hernia (8). Sometimes a scrotal hernia contains a long pelvic vermiform appendix. Therefore, symptoms of appendicitis may be similar to orchitis or testicular torsion (8, 38). Knowing the atypical symptoms due to the variable length and localization of the tip of the appendix can help prevent misdiagnosis and minimize the delay in administering appropriate clinic treatment (8).

The anatomical variability of the mesoappendix is usually associated with the absence of the mesoappendix. However, some studies in the literature have shown that short mesoappendix can cause acute appendicitis (20).

Appendix Duplication

Although it is a very infrequent congenital abnormality, misdiagnosis and mismanagement of such cases can lead to poor clinical outcomes and serious medical complications (39). Duplex appendix cases may also have legal consequences, particularly in individuals who have had appendectomy surgery (40).

The etiology of appendix duplication or horseshoe-shape appendix is still uncertain (39). The incidence of vermiform appendix duplication is 0.004% (1, 41). The duplications are divided into three categories: partial duplication of an appendix ordinarily found in the cecum (Type A), two separate appendices in a single cecum (Type B), and duplication of the cecum, each with its own appendix (Type 3) (1, 42).

Type B is also divided into two subgroups. Group B1 contains two symmetrically localized appendices on both side of the ileo-cecal valve. This group was named bird-like or avian type because it resembled the standard phylogenetic arrangement in birds. Group B2, also called taenia-coli type, has a normal localized vermiform appendix originating from the cecum in the normal region. In addition, there is a separate, primitive vermiform appendix localized along the taenia line. Type C has two cecum, each with its own appendix. It is difficult to classify some cases into a appropriate type, so the researchers began to add new types to the classification. Horseshoe vermiform appendix with two openings in the common cecum was classified as Type D (13).

Most appendix duplications are diagnosed during surgery or postmortem examination. It usually causes no symptoms, but symptoms can occur if the organ becomes infected or obstructed (41). Kahramanca et al. presented a case of B2 type vermiform appendix duplication in a 24-year-old male patient during emergency laparotomy (43). Bolatkale et al. presented a case of double vermiform appendix in the localization of the cecum using non-contrast helical abdominopelvic CT (39). In another study, double appendices, normal and accessory, were detected in a 56-year-old male cadaver during dissection. Accessory appendix was located at the ileocecal junction along the taenia line (B2 type). It was smaller than the normal appendix and measured 2.6 cm from base to tip. There was a mesoappendix containing appendicular artery on the free edges of both appendices. Histologically, both appendices contained normal mucosa, submucosa, muscularis externa, and serosa (13).

Triple Appendix

As stated before, appendix anomalies are extremely rare malformations. But rather than appendix duplication, Uriev et al. presented in their study a type of triple appendix in the microscopic examination after appendectomy. In the case, acute perforative appendicitis was detected due to the formation of three separate appendiceal lumen. These three appendix lumens were separated from each other by the submucosa layer and were located within a sole muscle layer (44). In another case report in the literature, a triple vermiform appendix was presented in a boy with serious congenital anomalies of the double penis and ectopia vesicle during laparotomy for the ureteroileal canal (45, 46).

Agenesis

Congenital agenesis of the vermiform appendix is quite infrequent. It is a condition that is found incidentally in cases with suspected appendicitis and performed laparotomy, and its frequency is approximately 1/100.000 (47).

When Collins examined the appendix by laparotomy in 50,000 individuals, he observed eight cases of malformations, including four agenesis and four duplications, and made a special classification for it. According to this, Collins categorized appendiceal malformations as follows. Absence of both cecum and vermiform appendix is classified as Type I. Type II refers to the absence of a primitive cecum and vermiform appendix. Type III has a normal cecum and no vermiform appendix. Type IV refers to a normal cecum and a rudimentary appendix, and presence of a giant cecum and absence of vermiform appendix are categorized as Type V (20, 48).

In the article by Vieira et al. in which they evaluated 37 studies, appendicular duplex related with acute appendicitis, mainly Type B2, followed by Type III agenesis were the most common abnormalities. The first case of agenesis connected with acute appendicitis and volvulus-type duplication has also been reported. In the same study, the highest rate was in the retrocecal position in adults, while in the pelvic position in children (20).

Concerning the surgical operation, some researchers recommend that in the absence of the appendix, the whole cecum should be immediately mobilized and followed up to the site of taenia coli. The ileum and retrocecal fields should be examined in detail before agenesis is diagnosed (49).

Diverticulosis of The Appendix

Small pockets of the mucosa and submucosa layers can pass through weaknesses in the appendicular wall, making the vermiform appendix susceptible to diverticulosis. The incidence of these cases is 1.4% (48).

Diverticulosis of the appendix is divided into acquired false diverticulum or, although very rare, true congenital diverticula. The diverticulum must be differentiated from a partial copy of the appendix. This is a remnant of a seconder and temporary appendix. These blunting occurs when the embryo reaches 20 mm in length (50). False diverticulum is usually diagnosed when a blockage occurs. Thus, etiologically, it is assumed that there is a rise in intraluminal pressure in the muscularis propria layer, usually eventuating in herniation of mucosa through a defect or weak area in a penetrating artery area (51).

Diverticulosis is often asymptomatic; however, in the case of diverticulitis, it can cause right lower quadrant pain like that felt in appendicitis. However, in the case of diverticulitis, the probability of perforation of the vermiform appendix may increase by 66%. It can also increase the death rate from acute appendicitis by 30 times. Owing to these possible consequences, the possibility of diverticulitis of the vermiform appendix should also be taken into account in patients suffering from lower abdominal pain (1, 52). Appendiceal diverticulosis affects the elderly (mean 60 years) more frequently than young people. Clinical symptoms occur more slowly compared to acute appendicitis (50).

Appendicitis

Appendicitis is the most frequently acute surgical situation in the abdomen and occurs in approximately 7% of the population (1, 12). Acute appendicitis is mostly caused by obstruction and follows a pathogenesis similar to other hollow viscera. Occasionally a gallstone, feces, tumor, or worms block the appendix, causing increased intraluminal pressure as well as venous outflow insufficiency (2). Its narrow blind-end lumen, plenty amounts of lymph tissue, and its anatomical structure, which tends to be clogged with fecaliths, predispose the appendix to inflammation (1, 12). At younger ages, this obstruction is mostly caused by lymphoid hyperplasia. Ischemic injury occurs when the intraluminal pressure exceeds the perfusion pressure. This encourages bacterial overgrowth and triggers the inflammatory response. Perforation of the infected appendix is a surgical emergency, as bacterial contents can leak into the abdominal cavity (1, 2, 12, 53). Unperforated appendicitis has a mortality rate of less than 1%. In patients with delayed diagnosis, the death rate may increase by 5% or more and the risk of perforation increases (54). When the vermiform appendix wall becomes infected, the visceral afferent fibers are stimu-

lated and enter the spinal cord at the T8-T10 level, causing widespread periumbilical pain and nausea seen in appendicitis. Advanced inflammation irritates the parietal peritoneum and stimulates somatic nerve fibers, causing localized pain. The location of the tip of the vermiform appendix indicates where the pain is localized. For example, the retrocecal position may cause right flank pain. The already existing right lower quadrant pain increases when the leg of a patient lying on his left side is placed in the extended position. The reason for this is the stretching of the right iliopsoas muscle adjacent to the vermiform appendix (Psoas Sign). Another important finding detected during physical examination in appendicitis is McBurney sign. When the abdominal wall is palpated in appendicitis, pain, tenderness, and defense occur at the McBurney point (two-thirds the distance from the umbilicus to the right anterior superior iliac spine). However, these signs and symptoms may not always be present, making it difficult to confirm the diagnosis of appendicitis. Clinically, the patient generally has symptoms such as nausea, vomiting, low fever and slightly elevated white blood cell count (2).

Appendicitis in Pregnancy

One of the most often non-obstetric surgical emergencies in pregnant women is appendicitis (55-57). Diagnosis may be difficult due to the appearance of signs and symptoms overlapping with appendicitis and the restricted use of radiological methods in pregnancy (58). Especially as pregnancy progresses, diagnosis may become more difficult in pregnant patients. This was partially attributed to the migration of the vermiform appendix from its normal position in the abdomen as the uterus increases in size (55). Reported complications from an undiagnosed appendicitis; wound infection, sepsis, preterm labor, preterm uterine contractions, preterm delivery and ileus pneumonia (59). The development of appendicitis in a pregnant patient can be dangerous both mother and fetus. In the literature, fetal morbidity and mortality rates for uncomplicated appendicitis have been reported as 1.8% and appendicular peritonitis rates up to 35% (60). 70% of vermiform appendix perforations come about in the third trimester (55, 61). Therefore, appendix localization is also important during pregnancy. Gradual upward displacement of the vermiform appendix during pregnancy was detected using magnetic resonance imaging method, but no upward displacement was observed in a research using direct surgical investigation (55, 56). It was observed that the rates of localized pain in the right lower quadrant ranged from 25% to 90%, and the rates of pain in the right upper quadrant ranged from <10% to 42% (55, 60). In House's study of pregnant patients with abdominal computed tomography (CT), the vermiform appendix was increasingly located in the right upper quadrant as pregnancy progressed. The rates of localization

in the right upper quadrant were 36% in the second trimester, and 72% in the third trimester. In addition, the vermiform appendix was seen just lateral to the right kidney in one patient in the third trimester. According to the results of this study, the vermiform appendix may not be present in the right lower quadrant in the later stages of pregnancy. As the uterus enlarged, the probability of the appendix being located in the right lower quadrant decreased, while the rate of being located in the right upper quadrant increased (55).

Appendectomy

Appendectomy surgery has been performed in the treatment of acute appendicitis for more than a century (12). McBurney first described this treatment in 1894. The McBurney incision technique was used as the primary technique until Semm developed the laparoscopic technique in 1980 (1). In the traditional appendectomy performed as an open surgery, an incision is made over the McBurney point. The McBurney point indicates approximately where the appendix attaches to the cecum. This point is at the 1/3 outer lateral point of the imaginary line between the right anterior superior iliac spine and the umbilicus. A surgical operation is started by making an incision at a right angle to this line at the McBurney point. One-third of the incision is made above this point and two-thirds below this point. After entering the abdominal cavity, the cecum is used as a guide. First, the location of taenia coli is determined, and then the location of the vermiform appendix is found by following the bottom part of the appendix. The basis of the appendix is located at the junction of the taenias. That's why this point is considered a handy anatomical landmark (1, 3). Appendectomy surgery performed with the laparoscopic technique has significant advantages over open surgery (54). Compared with open appendectomy, postoperative complications such as wound infection and ileus were observed to be less common in patients who underwent laparoscopic appendectomy. Various advantages such as early postoperative mobilization, small wound, less analgesic and antibiotic use, and shorter hospital stay have been demonstrated (62). However, it is stated that the laparoscopic technique is generally more costly, requires a longer operation time (54), and the rate of intra-abdominal abscess formation is higher (63).

Conclusion

The vermiform appendix has been a source of controversy over the years as an organ that appears insignificant but can often cause life-threatening complications. Recent advances in immunological research have supplied some proof for the function of the vermiform appendix. Advances in surgical and diagnostic techniques such as laparoscopy, ultrasonogra-

phy, computed tomography and laboratory tests have led to a considerable decrease in the acute appendicitis death rate. Although appendectomy is a classical surgical procedure, the unusual location and anatomical variations of the vermiform appendix can be challenging even for experienced surgeons (12). It is substantial for surgeons to have a detail knowledge and comprehension of the appendix. Embryological, anatomical and congenital variations of the appendix should be considered when deciding on the correct clinic diagnosis and treatment. Having this knowledge is also important in terms of contributing to the education of healthcare professionals.

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Chapter 8

THE ROLE OF CRISPR/CAS SYSTEM IN HEALTH SCIENSE AND RECENT ADVANCEMENTS

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1. Introduction

In our world mankind is frequently exposed to various mutagen factors due to industrialization, consuming unnatural food and chemical products, infecting by external microorganisms and viruses etc. While many genetic alterations consist of these kind of factors, various disorders threatening our world with causing a great amount of deaths are observed (Khan, Khan, Suleman, Zahid, & Nabi, 2015). Furthermore the variety of these disorders has a broad range from nervous system diseases such as Huntington disease (McColgan & Tabrizi, 2018), viral infections such as HIV (Pandey, Chouhan, & Verma, 2017) to many different types of cancers on different regions of the human body (Hassanpour & Dehghani, 2017). One of the most prominent aims of the biotechnological research area is developing effective therapeutic agents and diagnosing tools against the related genetic disorders. In this scope many chemical drug molecules targeting related genetic alterations and other micro or macro level solutions have been developed so far (Sun, Hou, & Zhang, 2014).

Besides those, as a treatment approach, manipulation of various natural mechanisms has come into prominence with their higher specificity. RNA interference (RNAi) is one of the main approaches that have been developed in order to reduce expression level of the product of genetic sequence carrying related alteration. RNAi is a kind of natural post transcriptional regulation mechanism and the mRNA expression level is regulated by the cells containing RNAi components (Rao & Sockanathan, 2005). During the manipulation method double stranded synthetic short RNA molecules (20-25 nt) such as silencing RNA (siRNA) and short hairpin RNA (shRNA) are delivered to the cell containing the related mutation. Once that RNA molecule are processed by Dicer enzyme and the antisense strand is loaded into the RNA induced silencing (RISC) complex. Owing to its sequence specificity, mRNA product is recognized by antisense strand and cleaved by RISC complex. Thus the silencing of genes in mRNA level has been completed (X. Chen et al., 2018). Due to the RNAi provides transient silencing, editing the sequence at the genomic level is required for achieving permanent results. As such, various other approaches which are designed as nuclease enzymes constructed with DNA binding domains and cleavage domain of a restriction enzyme have been developed such as Zinc Finger Nucleases (ZFN) (Urnov, Rebar, Holmes, Zhang, & Gregory, 2010) and Transcription activator-like effector nuclease (TALEN) (Becker & Boch, 2021). Nevertheless their less efficiencies limit the usages of them as therapeutic agents.

However, by the discovery of CRISPR/Cas systems, the requirement for mechanisms that might be manipulated as genome editing has been compensated considerably. Mojica and his colleagues have discovered

special repetitive sequences within the genome of Haloferax mediterranei –archae with high salt tolerance- isolated from marshes of Spain's Santa Pola coasts and named this sequences as clustered regularly interspaced palindromic repeats (CRISPR). Thus the CRISPR sequences have been identified for the first time in 1993 with this research (F. J.M. Mojica, Juez, & Rodriguez-Valera, 1993). Once that, these special repetitive sequences have been revealed within the genome of distinct organisms such as Yersinia pestis (Pourcel, Salvignol, & Vergnaud, 2005), Clostridium difficile (McAllister & Sorg, 2021), Mycobacterium tuberculosis (J. Wei et al., 2019) etc. Following researches revealed that these repetitive sequences serve for special pathways with the help of other special sequences which are called CRISPR associated (Cas) genes. In particular, once Cas enzymes could cleave targeted sequence by loading of special sequences produced by CRISPR loci had been enlightened, the idea of the usage of this mechanism as gene editing tool was born (Ishino, Krupovic, & Forterre, 2018). At this stage, lots of CRISPR based therapeutic and diagnosis applications as well as tools for investigating the function of related genomic sequences have been developed and utilized with their significant advantages. As such, the working principle of CRISPR/Cas systems as both natural mechanism and manipulation method, various application areas in biotechnology and recent developments have been introduced with this document.

2. Types and Function of CRISPR/Cas Systems as Natural Mechanism

In their following study Mojica and her colleagues had discovered some other special genomic patterns which are named as spacer amongst CRISPR loci within genomes of microbial organisms (Francisco J.M. Mojica & Rodriguez-Valera, 2016). Afterwards those spacer sequences were extracted from E.coli genome and analysed by using DNA databases. Interestingly, results demonstrated that these spacer sequences are also found in the genome of P1 phage which is an E.coli infecting virus strain (Luo et al., 2016). According to this finding, they have hypothesised that this special CRISPR loci and spacers could serve as adaptive immunity against viruses in their study which was published on Journal of Molecular Evolution (Francisco J.M. Mojica, Díez-Villaseñor, García-Martínez, & Soria, 2005).

Further publications have proven this theory and revealed that CRISPR spacer sequences found within bacterial genome are acquired DNA segments during previous viral infections (Barrangou & Marraffini, 2014). Moreover while these sequences are functional to provide immunity against viruses, this immunity is transferred to the next offspring of bacterial strains. The immune process which is promoted via CRISPR/Cas

systems might be summarized as; i) Adaptation: to process of viral DNA during infection and insertion those fragments by Cas1 and Cas2 enzymes amongst CRISPR loci found in host genome or plasmids, ii) Expression: transcription of these sequences as precursor CRISPR RNA (pre-crRNA) and processing it to mature CRISPR RNA (crRNA) molecules, iii) Interference: addition of the crRNA into Cas enzymes, targeting and degrading of viral genes by Cas/crRNA complex with sequence specificity (Figure 1) (Sorek, Lawrence, & Wiedenheft, 2013).

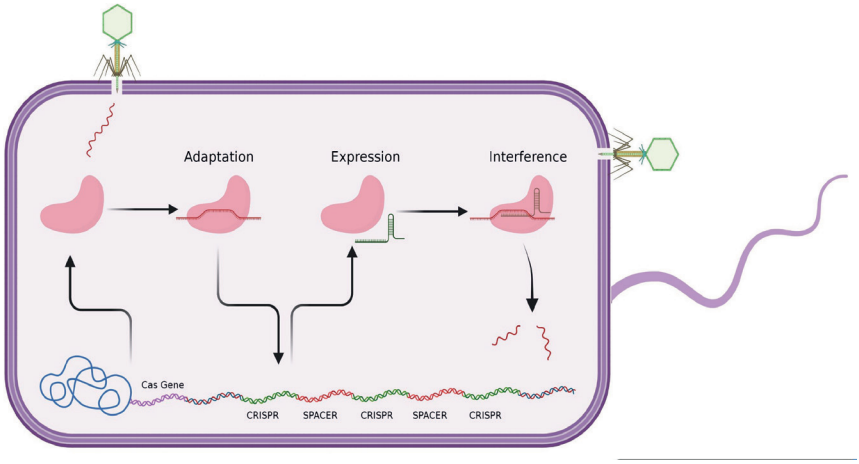

Figure 1. *Principles of CRISPR/Cas system as immune mechanism of bacterial organism against viral infection.*

In the last decade many distinct CRISPR/Cas systems have been identified over various organisms. While in common all those systems work as immunity against viruses, main differentiations are depending on localizations of CRISPR sequences, Cas gene contents, and action mechanisms of the pathways. As such three main CRISPR/Cas systems and subclasses have been identified as; Type I-A, Type I-B, Type I-C, Type I-D, Type I-E, Type I-F, Type II-A, Type II-B, Type II-C, Type III-A, Type III-B (Zhenquan Liu, Dong, Cui, Cong, & Zhang, 2020).

Due to its convenient mechanism, Type II is the most common type of CRISPR/Cas systems in usage as genome editing tool (Tang & Fu, 2018). There are only four Cas gene segments found in CRISPR regions of the genome which are Cas1, Cas2, Csn2, and Cas9. Apart from them, a non-coding RNA molecule with the name of trans-activating Crispr RNA (tracrRNA) is transcribed from CRISPR loci and combined with other non-coding RNA molecule with the name of Crispr RNA (crRNA) via Watson and Crick base pairing. This tracrRNA-crRNA complex is called as guide RNA (gRNA). Once that gRNA has been created, it is loaded into Cas9 enzyme –which is a specialised nuclease enzyme– and therefore

Cas9/gRNA complex recognizes the related sequence and creates double strand break (DSB) through Cas9 activation onto foreign DNA molecule (Mir, Edraki, Lee, & Sontheimer, 2018).

3. Application Areas of CRISPR/Cas Sytems in Health Sciences

3.1. Genome Modification

Contrary to gene silencing in mRNA level with RNAi technique, genome editing should be performed for many genetic disorders for permanent results. However the low efficiencies of ZFN and TALEN techniques trigger scientists to develop more efficient methods in genome editing. As such, manipulation of CRISPR/Cas systems has come into prominence, and many various approaches have been developed during several years (Nidhi et al., 2021). Nevertheless all genome editing techniques are depending on creation DSBs over specific sequences of the host genome (Hongyi Li et al., 2020). Afterwards those DSBs are repaired by natural repair mechanisms. Two distinct repair mechanisms are functional in this process which are non-homologous end joining repair mechanism (NHEJ) and homology directed repair mechanism (HDR). While NHEJ is repairing DSB via insertion or deletion of a few bases (indels) (Chang, Pannunzio, Adachi, & Lieber, 2017), HDR repairs DSB by usage homology armed template oligonucleotides (Davis & Maizels, 2014). Thus, by manipulating these natural repair mechanisms, the desired mutations could be created over the host genome.

In principle, genome modification via CRISPR/Cas system is based on delivery of an engineered RNA molecule which is named as single guide RNA (sgRNA) mimicking gRNA and Cas9 enzyme (Allen, Rosenberg, & Hendel, 2021). Due to that Type II CRISPR/Cas system is manipulated in this process, Streptococcus pyrogenes Cas9 (SpCas9) is frequently used for creating DSB on host DNA (Ran et al., 2013). The regular CRISPR/Cas manipulation in genome modification is promoted through following steps; i) delivery of both sgRNA and Cas9 enzyme as either in molecule form or with expression agent, ii) creation of sgRNA/Cas9 complex by loading sgRNA into Cas9 enzyme, iii) recognizing related sequence ensuing protospacer adjacent motif (PAM) sequence –little nucleotide sequence (generally NGG or NAG)– by targeting with sgRNA, iv) creating DSB with cleavage activity of Cas9 enzyme, v) repairing DSB via manipulation of NHEJ or HDR mechanisms (Figure 2) (P. D. Hsu, Lander, & Zhang, 2014). It's demonstrated in literature that the system is working with superior specificity since that approximately 20 bp sequence which is adjacent to related PAM sequence according to Cas enzyme using as genome modification tool could be targeted with sgRNA molecule (Anders, Niewoehner, Duerst, & Jinek, 2014).

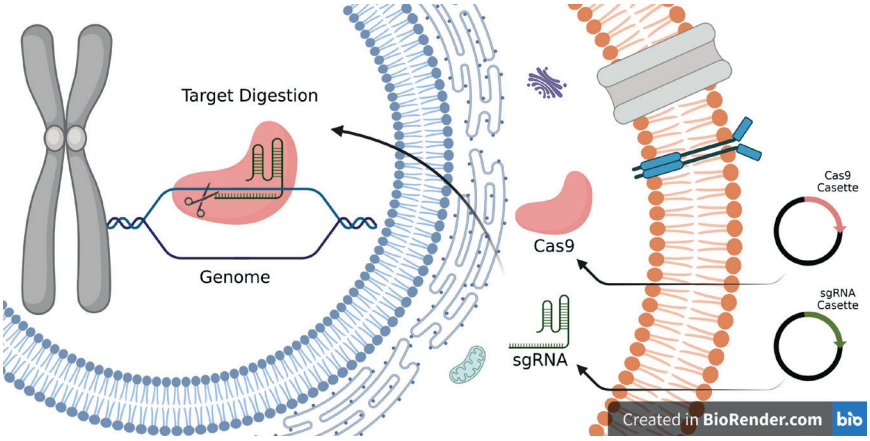

Figure 2. *Principles of genome editing with CRISPR/Cas9 technique.*

As such while many novel researches have been published with a broad range of applications in genome editing, the studies are still going on in order to develop innovative approaches. In order to analyze the efficiencies of CRISPR/Cas system and TALEN technologies according to one another Zhang and his colleagues have promoted a research. They have knocked myostatin (MSTN) gene out on Alpas cashmere goat with both CRISPR/Cas and TALEN systems. Their findings have demonstrated that electrotransfection efficiency of CRISPR/Cas application is 8.1% higher than TALEN. Moreover CRISPR/Cas cutting efficiency has been found to be higher as well. In addition MSTN-/- mutation frequencies have been observed 8.5 times more in CRISPR/Cas technique. In light of these results they have considered that the potential of CRISPR/Cas system is higher than TALEN as a gene engineering tool in farm animals breeding (Ju Zhang et al., 2019). In another study, Qin et al. have developed a CRISPR/Cas based method in order to edit the genome of Halomonas which is a halophile with industrial value. While they have constructed eight different mutants, they declared that the efficiency of their method reaches 100% (Qin et al., 2018). In the study completed in 2020, Oo and his colleagues have focused on the regulatory function of the Dip2C gene in brain tissue. They have modified the genome of brain cells by CRISPR/Cas system with 26.7% efficiency. The results of the study have shown that the role of the Dip2C gene in brain development and function is so significant since that it regulates the profile of many other genes. Besides the capability of CRISPR/Cas systems have been revealed on plants too (Oo et al., 2020). In the study which was carried out by Feng and his colleagues, the Zmzb7 gene had been targeted on the maize genome. Considering the findings they have declared that CRISPR/Cas systems efficiently work as a genome modification tool on both het-

erochromatic and euchromatic regions of maize chromosome (Feng et al., 2016).

3.2. Transcription Regulation and mRNA Silencing

Since that when DSB cannot be repaired with repair mechanisms, cells undergo genomic erosion and apoptosis respectively (Chatterjee & Walker, 2017). As another approach contrary to creating DSB on host genome, expression level of related genetic segments are regulated with CRISPR/Cas systems. In this scope, dead Cas9 (dCas9) which is a Cas9 derivative lacking of its cleavage activity has been produced through protein engineering techniques (Saifaldeen, Al-Ansari, Ramotar, & Aouida, 2020). Thus transcription of gene might be upregulated via CRISPR activator (CRISPRa) mechanism by guiding the transcription factor conjugated dCas9 (Becirovic, 2022) and downregulated via CRISPR interference (CRISPRi) by guiding dCas9 in order to inhibit the binding of transcription factor onto host DNA (Schultenkämper, Brito, & Wendisch, 2020).

Hsu et al. have published an article which is implying the significance of CRISPRa on bone tissue regeneration. In this study they have developed a CRISPRa system which could upregulate the expression of both Wnt10b and Foxc2 genes in order to activate canonical and non-canonical Wnt pathways. While the activation can last >14 days, the results demonstrate that osteogenesis has been promoted and adipogenesis has been repressed (M. N. Hsu et al., 2020). Similarly Li and his colleagues have developed a CRISPRa system providing synchronous transactivation of multiple long non-coding RNAs and transcription factors. They have declared that human induced pluripotent stem cells could be differentiated by this novel system to astrocytes and neurons (S. Li, Zhang, Xue, Li, & Liu, 2017). Besides these, different approaches depending on controlled activation of the gene expression regulation system is aimed to be developed. In a study carried out by Putri et al. the scientists have developed a CRISPRa system that can be triggered under blue light. Using this novel system they have arrived upregulation on ASCL1a, BCL6a, and HSP70 genes of zebrafish cells (Putri & Chen, 2018). Contrary to activation, genes could also be suppressed with CRISPRi mechanism. In 2020 Agarwal developed a novel CRISPRi system in order to repress several genes on the Mycobacterium tuberculosis genome. Promoting the research he has accomplished to suppress 10 different extracellular sigma factors with great importance for viability. Thus the study has opened a new gate in development of novel anti-tuberculosis drugs (Agarwal, 2020). Similarly, Depardieu et al. have developed a method including design rules and new vectors on CRISPRi technique in 2020. They have demonstrated that their developed method efficiently works to silence the gene on both S. aureus and E. coli genomes (Depardieu & Bikard, 2020).

Apart from CRISPRi, gene expression might be downregulated on mRNA level with distinct CRISPR/Cas systems. Similarly to the RNAi mechanism, in this approach mRNA molecules are silenced with sequence specificity. While other derivatives of Cas enzymes –such as Cas13a– are used for binding to mRNA molecules, they are guided with special sgRNAs designed as single stranded. Once that mRNA has been recognized, it is cleaved by Cas enzyme so that to be silenced (Kick, von Wrisberg, Runtsch, & Schneider, 2022; Junxia Zhang & You, 2020). For instance, Zhao and his colleagues have developed a Cas13a based technique silencing mutant KRAS mRNA molecules on pancreatic cells. While they have observed 94% knockdown efficiency, they reveal that the knocking down causes apoptosis of the cells in vitro (Zhao et al., 2018). In an intriguing study, Chen et al. have targeted mRNA products of E6 and E7 oncoproteins expressed by human papilloma virus (HPV). According to their results, the silencing of the related mRNAs brings growth inhibition about HPV16 and HPV18 positive cells as well as no effect has been observed on HPV negative cells. Moreover, reducing in tumor size and upregulation of tumor suppressor genes introduces the potential of Cas13a usage in treatment strategies on HPV sourced cervical cancers (Y. Chen et al., 2020). In another study, Kulkarni and his colleagues have achieved to silence vitellogenin mRNA product of Anophales gambiae and COPI mRNA α and δ subunits of Aedes aegypti via CRISPR/Cas13a application. They have reported that the developed system could efficiently works in order to silence several mRNA transcripts simultaneously with their results showing a reduction in egg production and differentiation on morphologies on two different mosquito species (Kulkarni et al., 2020).

3.3. Molecular Screening

During several years many molecular screening methods have been developed and used in order to detect the sequences on host genome or RNAs. For instance fluorescent in situ hybridization (FISH) is one of the most popular techniques which is applied by fixing the cells and recognizing the sequences by targeting with fluorescence labelled DNA probes (Hu et al., 2014). Moreover there are other approaches that are using fluorescent protein conjugated DNA probes for detection of the related sequence found in chromosome or RNA molecules (Boutorine, Novopashina, Krasheninina, Nozeret, & Venyaminova, 2013). CRISPR/Cas system is one of the significant candidates in screening the related sequences since that it has relatively superior specificity. During this application EGFP fused dCas9 enzyme is produced by protein engineering techniques and it is leaded by sgRNA designed to target sequence which is desired to be screened. Results are obtained by analysing the emission of EGFP (Ribeiro, Ribeiro, Barreto, & Ward, 2018). Apart from EGFP fused dCas9

enzyme, engineered Cas12a which is PAM labelled Cas enzyme has been produced and is used in detection of nucleic acid sequences (Ooi et al., 2021). Furthermore, in order to detect mRNA molecules, usage of Cas13a is another attempt manipulating in diagnosing several diseases such as cancer or viral infections (Barnes et al., 2020).

Chen and his colleagues have developed a technique using EGFP conjugated dCas9 in detection of repetitive and non-repetitive genomic sequences. Promoting this novel technique they have studied dynamics of telomere regions during elongation and disruption, MUC4 subnuclear localization, and its dynamics during mitosis. Consequently they have analyzed the efficiency of this technique on revealing chromosome dynamics of living cells (B. Chen et al., 2013). Wei has recently published a paper containing a significant application of the CRISPR/Cas12a system. In this study novel system has been developed in diagnosis of Staphylococcus aureus with high sensitivity. While the detection range has been determined as 106 to 102 CFU/ml, the system is desired to be used in detection of many other bacterial species due to its great selectivity and efficiency (J. J. Wei, 2021). Similarly Nguyen and his colleagues have developed a novel methodology with superior specificity. This detection system using Cas12a has been named as CRISPR-ENHANCE by the scientists. According to their declaration femtomolar levels of nucleic acids could be detected via this system either with fluorescence labelling detection or lateral flow assay (Nguyen et al., 2022). As another objective mRNAs could be detected by Cas13a usage. In the research conducted by Huang and his colleagues, the microRNA-17 (miR-17) family has been detected by a recently developed Cas13a based tool working with hyper branching rolling cycle principle. The tool has been named HyperCas and its sensitivity has been analyzed as superior according to conventional techniques in miRNA detection. Furthermore with its quite low limit-of-detection (LOD) it's considered a convenient tool in early diagnosis of various diseases (Huang, Huang, Yue, Shan, & Xing, 2020). In another study Sheng et al. have developed an electrochemical biosensor containing the dual application of CRISPR/Cas13a system with catalytic hairpin DNA circuit. According to their data they have achieved 0.952 specificity and 0.9 sensitivity in diagnosis of low expressing RNA molecules on non-small cell lung cancer (NSCL) progression. In light of these results they have reported that their electrochemical biosensor device exhibits sufficient performance for early diagnosis of NSCL with its low cost and great specificity (Sheng et al., 2021).

3.4. Gene Knock-In

In genome modification by using CRISPR/Cas systems, HDR is frequently manipulated natural repair mechanism in order to create desired

mutations into host genome. Recently scientists are using these application for permanent and inherited protein production in single cell with a mechanism naming knock-in (Petazzi, Menèdez, & Sevilla, 2020). Once that DSB has been created by sgRNA leaded Cas9 enzyme, it is repaired by HDR mechanism by insertion the template sequence expressing related protein. In general this system is depending on co-transfection of three plasmids which are responsible for sgRNA transcription, Cas9 expression, and template sequence introducing into single cell. Thus the protein expressing sequence is inserted into host chromosome and it is transmitted to offspring during proliferation (Nidhi et al., 2021).

In a study completed by Lo and his colleagues, they have accomplished to create a cell line expressing recombinant proteins by inserting transgenes into chromosomes through CRISPR/Cas9 technique. They aim to use this novel technique in displaying the expression level of recombinant protein into cells (Lo, Greben, & Chen, 2017). In another study, Antonova et al. have successfully achieved to insert eGFP into chicken chromosomes under the control of GAPDH gene promoter by developing a CRISPR/Cas9 system. Furthermore they have highlighted that the developed system could be used in creating genetically modified birds (Antonova et al., 2018). Similarly, Kanca and his colleagues have adapted their previous CRISPR/Cas9 method used in to knock-in of Drosophila genes introns to inserting GFP tagged proteins marking S2 cells' organelles in 2019. Besides, they have evaluated their method as cheap, fast, and scalable (Kanca et al., 2019). In a comparison study, Liu et al. have analyzed the knock-in efficiencies of ZFN, TALEN, and CRISPR/Cas9 systems. They have tried to knock-in of hFat-1 and eGFP genes in bovine fetal fibroblasts and goat fetal fibroblasts. The results demonstrated that while hFat-1 knock-in efficiencies in bovine fetal fibroblasts were 0%, 79.01% the eGFP knock-in efficiencies had been recorded as 13.68%, 77.02% for ZFN and CRISPR/Cas9 systems respectively. Furthermore, the hFat-1 knock-in efficiencies in goat fetal fibroblasts had been identified as 26.47%, 74.29% and eGFP knock-in efficiencies were 32.35%, 70.37% for TALEN and CRISPR/Cas9 systems respectively. These results prove the greater efficiency of CRISPR/Cas systems according to ZFN and TALEN techniques in knock-in processes (LIU et al., 2018).

3.5. Therapeutic Applications

From the origin of requirements such as genome modification tools, mankind suffers from several genetic disorders. While CRISPR/Cas had been evaluated as an efficient tool for genome modification, its efficiency over genetic diseases was revealed with many researches. In particular, significant progresses has been made on cancer disease by the CRISPR/Cas system (Katti, Diaz, Caragine, Sanjana, & Dow, 2022). Since that

cancerous tissues might be formed with many factors such as activating oncogenes, inactivating tumor suppressor genes, and disorders on epigenetic modifications, various applications have been developed against these factors in literature. In these applications mutations causing the oncogenes activation, tumor suppressor gene inactivation, and deficiencies on enzymes responsible for methylation or acetylation of histone proteins could be repaired (Fan et al., 2018; Yin, Xue, & Anderson, 2019). As an instance for CRISPR/Cas9 applications on cancer therapy, Li and his colleagues have completed a research investigating HIF-1α downregulation with CRISPR/Cas9 technique in order to regulate tumor microenvironment. In this study scientists have succeeded to encapsulate plasmids expressing Cas9 and sgRNA targeting HIF-1α into peptide mediated liposome delivery systems. According to their results, downregulation on expression of HIF-1α and its downstream products such as MMP-9 and VEGF has been observed to lead quite antimetastatic effects. Moreover they have revealed that the developed strategy suppresses metastasis of pancreatic cancer, and reported that this technique might be improved to be a novel approach for cancer therapy (M. Li et al., 2019). In an alike research Dong et al. have tried to silence HIF-1α with CRISPR/Cas9 but in macrophages in order to create tumor suppressive microenvironment. While they silence HIF-1α epigenetically via methylation of histone H3 on its promoter region, they have shown that macrophages could be reprogrammed with tumor suppressing phenotype and tumor angiogenesis could be inhibited with their strategy (Y. Dong et al., 2021). In another study Zhang et al. have silenced PDEF acting significant role on tumor progression in the SGC and AGS gastric cancer cell lines. Through this application they have observed inhibition on migration, invasion, and cell proliferation of the cancerous cells. They have declared considering the results that novel treatment therapy for gastric cancer could be developed with this technique (Y. Q. Zhang et al., 2019). Shi and his colleagues in 2017 have accomplished to knock out the CTLA-4 protein which is evaluated as tumor inhibitory checkpoint during tumor progression with CRISPR/Cas9 technique. While until this research CTLA-4 had been blocked with many inhibitory ligands, the scientists observed over 40% reduction in cancer cell viability by their permanent silencing approach. Furthermore, an increase in the expression of TNF-α and IFN-γ was achieved with CTLA-4 KO. Consequently they have developed an efficient method significantly increasing antitumor activity of cytotoxic T lymphocytes (CTL) (L. Shi et al., 2017). As a similar approach Deng and his colleagues have analyzed downregulation of PD-L1 which is another immune checkpoint via silencing Cyclin-dependent kinase 5 (Cdk5) with CRISPR/Cas9 technology. The results have demonstrated that tumor microenvironment could be reprogrammed as including strong T-cell mediated response to

cancerous cells, tumor growth could be inhibited on triple negative breast cancer and murine melanoma, and the PD-L1 downregulation through CRISPR/Cas9 could be developed as next generation treatment strategy for cancer disease (Deng et al., 2020).

In addition, such those applications are tried over infectious diseases as well. Most attractive developments in these areas are observed on human immunodeficiency virus (HIV) infections. Due to that HIV being a kind of retrovirus, and infecting the immune cells via recognizing their CCR5 and CXCR4 surface receptors, mutations are able to be created with manipulation of CRISPR/Cas systems and HDR mechanism on the genes expressing those receptor proteins. Therefore the cells are protected against HIV infection by blocking the interaction with CCR5 or CXCR4 (Z. Zhang, Hou, & Chen, 2022). Meanwhile this approach is often tried over other viral infections. In this scope during the study conducted in 2017 Liu et al. regulate the expressions of both CCR5 and CXCR4 simultaneously. In this study while two different sgRNAs had been designed for each target, both were delivered to cells within a single vector. Furthermore, while no toxicity had been observed during treatment, it was reported that the system might be improved as a safe HIV-1 curing strategy (Zhepeng Liu et al., 2017). In another research Ashraf and his colleagues have developed a novel method based on CRISPR/Cas13a in order to target single stranded RNA hepatitis C virus (HCV). They have designed Cas13a variants that could recognize conserved regions of HCV genome with computational methods. In the light of results they have demonstrated that HCV replication could be inhibited with no toxicity on the huh-7.5 cell line (Ashraf et al., 2021). In another similar study, Li et al. have developed a CRISPR/Cas13a system to inactivate dengue virus in mammalian cells. Consequently they have declared that this novel method could be used on treatment of dengue virus and other RNA viruses (Hao Li et al., 2020).

In our era, specialized treatment approaches are evaluated as promising over a broad range of genetic diseases. For instance, Car-T cell (Sterner & Sterner, 2021) and induced pluripotent stem cells (iPSC) strategies (Y. Shi, Inoue, Wu, & Yamanaka, 2017) depending on extraction of the cells, modification of the genome, and reinjection to the host body are the most common primary approaches in specialized treatments. It's demonstrated that during the genome modification step, CRISPR/Cas systems could be used with high efficiency in these treatment strategies (McTague, Rossignoli, Ferrini, Barral, & Kurian, 2021; Razeghian et al., 2021). For instance Burnight and his colleagues have accomplished to restore various genes causing retinal degeneration by using HDR dependent CRISPR/Cas systems on patient-derived induced pluripotent stem cells (iPSC)

(Burnight et al., 2018). In addition, the efficiency of CRISPR/Cas systems has been proven on other genetic diseases such as Duchenne muscular dystrophy (DMD) (Choi & Koo, 2021), cystic fibrosis (CF) (Maule, Arosio, & Cereseto, 2020), sickle cell anemia (Frangoul et al., 2021) etc.

4. Improving the Efficiency

Although CRISPR/Cas systems are evaluated as recently efficient tools used in genome modification and other biotechnological research areas, few limitations encountered in these systems still should be overcome. Off-target effect which is described as nonspecific DSB creation over host genome is one of these limitations (Zischewski, Fischer, & Bortesi, 2017). While this phenomena is observed on other techniques such as RNAi, ZFN, and TALEN, CRISPR/Cas is evaluated with higher specificity since that the genomic segments are targeted by approximately 23 nucleotides (20 nucleotides adjacent to 3 nucleotides PAM sequence are recognized by sgRNA loaded Cas enzyme) (Gaj, Gersbach, & Barbas, 2013). Nonetheless it's discovered that this specificity should be progressed. As such various bioinformatics tools and software that might be used for designing proper sgRNAs have been developed for several years. For instance, Elevation, Benchling, Azimuth, CHOP-CHOP, CRISPRscan, GuideScan are most common tools providing sgRNA designs with great specificity and efficiency (Brazelton et al., 2015; Cui, Xu, Cheng, Liao, & Peng, 2018).

Furthermore, it's revealed that specificity could differ according to Cas enzyme derivatives and concentration. In particular, that the modifications on undesired regions of the host chromosome are more frequently encountered on the cells exposed to extra Cas enzyme has been recently discovered. Therefore, it's considered that the time interval of Cas enzyme existance in cells should be limited in order to inhibit creation of undesired DSBs (Uddin, Rudin, & Sen, 2020; Yang, Xu, Ge, & Lai, 2021). In this scope novel recombinant Cas enzymes with low half-life and stability have been produced and used in many research. Thus once that desired DSB has been created by Cas enzyme, it is degraded and its mechanism is blocked for inhibiting creation of undesired modifications. Besides, other Cas enzymes created with distinct side groups are recently produced with improved specificity such as HypaCas9, eSpCas9 (Ikeda, Fujii, Sugiura, & Naito, 2019; Slaymaker et al., 2016) etc. In addition, it's revealed that the specificity could be improved by considering the PAM sequences. As such in many research, Cas enzymes have been modified in order to recognize different PAM sequences such as NGA, NGG, GAA, NG, and GAT (W. Zhang et al., 2021).

As another concept to reduce off-target effect, targeting the genomic segment from two distinct regions promises in genome modification ap-

plications. As such, novel sgRNA:Cas9 complex naming double-nicking (dnCas9) has been developed. In this technique the genomic segment is targeted from two different regions on distinct strands via two Cas9 enzyme engineered for creating the nicks on DNA strands. Furthermore, while the first sgRNA has been designed in order to recognize upstream sequence, the second sgRNA could recognize downstream sequence of the genomic region that is desired to be modified. Therefore nicks are created on the regions flanking the genomic segment via nickase Cas enzymes and repaired by HDR mechanisms with introducing template sequences (Figure 3) (Ran et al., 2013).

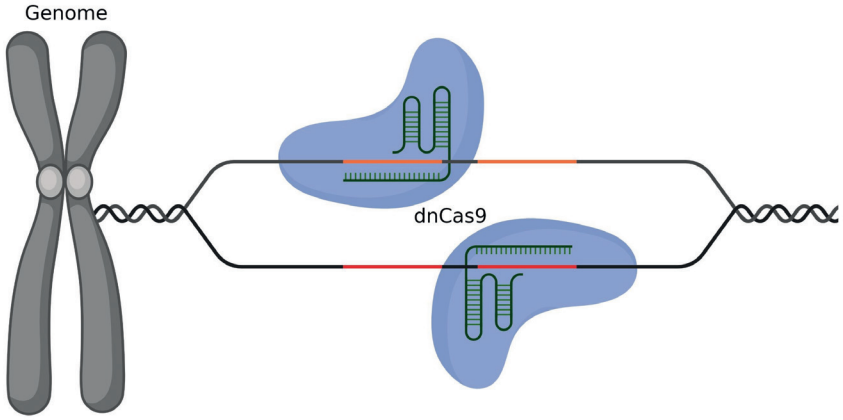

Figure 3. *Targeting the genome over two distinct regions with dnCas9 in order to increase specificity of the technique.*

Apart from the specificity, delivery of molecules that are used in CRISPR/Cas systems is another limitation. In general sgRNAs and Cas enzymes are delivered to the cells within distinct plasmids. However the sizes of this kind of vectors do not enable ease in the delivery processes (Moore, 2001). This phenomena forces the scientist transmitting the sgRNAs as naked and Cas9 in protein form (Lino, Harper, Carney, & Timlin, 2018). However other limitations in delivery of such biomolecules still keep its importance in this kind of applications. For instance, the biological barriers such as content of blood plasma, endo-exonuclease enzymes, undesired electrostatic interactions between negatively charged cell membrane and negatively charged nucleic acids, and high acidic conditions in endosome vesicles are main problems during delivery of biomolecules used in gene therapy approaches (Pan et al., 2021; Sung & Kim, 2019). One of the most prominent efforts of the biotechnological research area is basing on to develop delivery agents in order to transmit the biomolecules to the cells with high efficiency and by escaping from biological barriers (Tiwari et al., 2012). As such, various both viral vectors such as adeno-associated viruses (AAV) (A. Li et al., 2020), lentiviruses (LV) (W. Dong &

Kantor, 2021), and retroviruses (RTV) (Knopp et al., 2018) and non-viral vectors designed with polymers (Jo et al., 2020), lipids (Qiu et al., 2021), and inorganic compounds (Qiu, Glass, & Xu, 2019) have been synthesized and analyzed according to their transfection efficiency, toxicity, and immunogenicity parameters.

5. Conclusion

Last decades have witnessed too much development in nucleic acid based strategies as treatment and diagnostic approaches for various diseases. Among these techniques, manipulation of RNAi mechanism, ZFN and TALEN tools have been considered as promising, yet the drawbacks of them has forced the scientists so that they discover more proper and great efficient mechanisms. CRISPR/Cas systems have been evaluated as primary candidates with their convenient principles. While those systems are discovered as natural immune process of various microorganisms against viral infections, different types have been classified during several years. However, that Jennifer Doudna and Emmanuelle Charpentier have been awarded the Nobel Prize in Chemistry in 2020 since they developed a reprogrammable gene editing system is highlighting the significance of CRISPR/Cas systems on biotechnological research areas. Major manipulation of the systems are encountered in genome modification. Furthermore, regulation of gene expression, molecular screening, recombinant protein production and therapeutic agent production for treatment of many genetic and infectious diseases are other application areas of CRISPR/Cas systems in health sciences. However it's highlighted that many limitations confine the application in the related objectives such as off-target effect and delivery problems. Nowadays off-target profiles of developed CRISPR/Cas strategies are improved by several bioinformatics tools and engineered Cas derivatives. In addition, various viral and non-viral delivery agents are produced in order to overcome delivery limitations. In light of these information the place of CRISPR/Cas systems in health sciences has been highlighted with this document.

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Chapter 9

NEW TRENDS IN DENTISTRY AFTER COVID-19: TELEDENTISTRY AND ONLINE PLATFORMS

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Teledentistry

Teledentistry is the use of electronic imaging, information and communication technologies to transfer and support consultation, diagnosis, information sharing, treatment and education.1 Teledentistry is not a new concept and one of the oldest teledentistry projects was established in the world by the US military to serve US troops in 1994.2 Over time, teledentistry has been proven useful in providing dental screening, diagnosis, consultation, recommendation of treatment planning and comparable to real-time consultations in areas with limited access to facilities, schoolchildren, and long-term healthcare facilities.3,4 The concept of teledentistry was first introduced in the literature in 1997 by Cook as “use video-conferencing technologies to provide diagnosis and advice about remote treatment”.5

The novel coronavirus-induced disease, which emerged in the city of Wuhan (China), has been named coronavirus disease-2019 [coronavirus disease-2019 (COVID-19)] by the World Health Organization (WHO) in the late December of 2019. The spread of the disease is by direct contamination, droplets and aerosols via face-to-face contact, sneezing and coughing.6 It was announced as pandemic on March 11, 2020 due to its occurrence in more than 110 countries and affecting densely populated areas of the world. The COVID-19 pandemic with its social, political, economic and psychological effects, has suddenly entered our lives and caused inevitable changes. Dentists are at risk of COVID-19 infection due to the use of sharp and aerosol-producing instruments, face-to-face contact, exposure to blood, saliva and other body fluids.7

After this pandemic, the effects of which still continue today, there have been undeniable changes in almost every field in dentistry, especially in examination, treatment practices and education. The pandemic necessitated taking measures to minimize the situations in which patients need to apply to the hospital in order to reduce personal contact, and thus, the tendency to online applications such as tele-health, telemedicine and teledentistry, which have been increasingly used in recent years, has increased. Teledentistry is a telemedicine branch in which telecommunication systems and health information technology used for consultation, oral-dental care, education and public awareness in order to improve oral and dental health.8 In a "teledentistry" search conducted on MEDLINE/PubMed as of July 2021, a total of 235 studies were found from the mid-1990s to the present and 16 studies have been brought to the literature in the last 5 years. This situation reveals that teledentistry is increasing its popularity day by day.7 In the COVID-19 pandemic and future similar pandemics and social crisis situations, it will always be imperative to meet the urgent and elective dental and oral treatment needs of the society.

The development of teledentistry, which has increased in importance after the pandemic, and its integration to the routine will be very useful in terms of possible health crisis in the future.

Teledentistry technologies enable physicians to access information by performing rapid transfer of images, videos and files. Messaging or video-conferencing programs that allow sharing patient information and communicating with patients, whether they are prepared on behalf of teledentistry or not, will make it possible to closely monitor the condition of patients and provide more patient-centered treatment. Teledentistry refers to applications that use information-oriented technology and communication systems to improve oral-dental health services.9

These applications can be used in all specialties of dentistry. Remote control of fast and recordable data, simultaneous communication with many people, reducing hospital costs and patient waiting times and therefore low health care costs in the long term are among the advantages of teledentistry.10 One of the biggest advantages of teledentistry is the potential to reach specialists in a timely manner, so reducing healthcare inequalities in oral care.8 Since teledentistry is a topic that has emerged in recent years, information about the effectiveness of these applications is limited.11 Future applications of teledentistry will allow for the opportunity to increase the use of oral and dental care services, reduce human resources and financial costs and improve health outcomes.8

The teledentistry interaction is categorized as asynchronic and synchronic. Information can be registered and sent for later analysis in asynchronic teledentistry (store and forward), while in synchronized teledentistry the interaction is real-time (e.g. videoconferencing). Adequate and necessary technical equipment to come together the dentists and patients without an examination environment, to provide sufficient quality information for diagnosis and to convey it in a quality manner in teledentistry: hardware (computer, mobile phones, video conferencing equipment, digital camera, diagnostic devices in dentistry), software (softwares to enable image acquisition, storage and transfer) and network connection.7

Terms used in teledentistry are: tele-consultation, tele-diagnosis, tele-treatment, tele-education, tele-application, tele-monitoring, tele-support, tele-management, access to patient medical information and mobile teledentistry.12 Tele-consultation is the remote consultation of the patient and healthcare personnel with the specialist and receiving treatment advice. Tele-education is the continuation of professional development at a distance. Tele-application is the remote practical training of healthcare professionals. Tele-monitoring is the regular remote monitoring of patients' vital signs and/or biochemical variables. Tele-support is the support

of remote healthcare facilities in isolated areas, distant locations, areas affected by armed conflict or natural disasters. Tele-management is the distant communication for executive tasks (such as appointment scheduling). Access to patient medical information allows the patient to access websites with static or interactive applications for both clinical needs and informing purposes. Mobile teledentistry is the use of mobile tools such as smart phones, electronic health records and portable radiography for purposes such as oral and dental health care, consultation, education and public awareness.12

Studies show that teledentistry is growing rapidly. With the development of technology, the current potential of teledentistry is also increasing. With the penetration of teledentistry into routine, the patient-physician relationship will change and perhaps health activities will be carried out remotely rather than just face to face. In this way, patients who have geographically limited access to health institutions, can not leave their homes due to chronic diseases or who have contagious diseases will be able to reach expert opinion easily. However, as far as we know, in the field of teledentistry, where very few studies have been done before, studies should be conducted on the cost-effectiveness of teledentistry, its use in long-term and large sample groups before these applications become widespread and these activities should be regulated by law.13,14

Recently, there has been a meaningful improvement in survival rate of patients with critical oral disease, and this outcome is directly related to the early diagnosis.15,16 Early diagnosis is the most effective way to reduce the individual burden of the disease, morbidity and mortality, and improve quality of life. For this to happen, healthcare professionals need to be close to their patients. Teledentistry is a promising application in terms of dental screening and triage, diagnosis, consultation and treatment plan in mandatory quarantine conditions together with pandemic and similar global epidemic situations.

Dentistry in Social Network and Social Media

Social media and social networks are platforms where people remark their thoughts on the internet and include applications that allow individuals to share information and express feelings in a comfortable way. Through the developing technology and widespread use of the internet, these digital platforms have become more powerful and important. Social media users take an active role in sharing and reporting news, while publishing reviews of health events other than public health context. 17 The WHO stated that social media can be used to catch public attention in emergency, simplify one-on-one communication, create situational awareness, observe and respond to rumours, society concern and reac-

tions".18 As WHO pointed out, it can be used as a quite powerful tool not just to reach information but also to gather feedback. Posts, comments and reactions of those who read the post are instantly seen and analyzed, their needs are understood and a faster feedback is provided.19,20

The ability and desire to social networking is a fundamental characteristic of being a human. For centuries, humanity has developed with social populations where individuals who are strengthened by the sense of belonging, share knowledge, opinions and experiences.As the world evolves in the matter of technology, social media defined as several internet-based applications that enable the formation and exchange of content by users and has become important to billions of people and affected almost every industry imaginable worldwide.21 In recent years, social tools have proliferated, providing new options to create content, connect, communicate and share information without necessitating extraordinary coding proficiencies.22 Social media platforms include wikis (eg. Wikipedia), social networking sites (eg. Facebook), blogs (eg. WordPress), status update services (eg. Twitter), social bookmarking (eg. Reddit) and media sharing sites (eg. YouTube).23 Studies have shown that 80% of internet users search for health information online.24,25 Social media is one of the favorite places to obtain health-related information and community support, and it has been shown to effect the healthy habits of peers above all else. Specifically, health-related information seeking, treatment and public health interventions, physical activity, signs and symptoms, diet/nutrition are among the leading online activities. There are questions about the certainty and objective nature of online health information, especially on user-generated content platforms. Also, an individual's internet proficiency may not correlate with medical literacy. Therefore, the position of social media in health services should not be ignored.26

On account of the COVID-19 pandemic, there has been a significant development of virtual dentistry services. Nevertheless this is still temporary and limited as there is no preparation for such a crisis.27 Although patients attend for online health information, the major part of masses stated that they would rather to receive it from physicians face-to-face. However, it is thought that patients mahe this choice because they cannot access health services by means of the social, economic, physical or cultural obstacles. Social media acts as a supporting network in terms of increasing patients' access to health information.26 One study found that supporting networks are the second choice after physicians for obtaining health information.28 With the integration of the social media in health services, more studies are needed for better understanding of the effects of social media on dentistry and enlighten dentists about how social media can be incorporated into dentistry. Contagious diseases such

as COVID-19 can cause behavioral, mental and emotional changes in society. Incalculable and unclear situations, being misinformed, severity of diseases and isolation conditions can exaggerate dental anxiety and fear. In recent years, analytics tools of social media have been used to observe public emotions and communication during public health emergencies such as Zika and Ebola outbreaks.29 Observing the social media posts of individuals in pandemics and similar global crises will help diminish feelings of fear and anger and reach informational messages suitable for the needs of the target audience.30 Healthcare services, especially during crisis and epidemic/pandemic, necessitate the rapid and effective spreading of information to physicians, patients and society. By assuming a more active social media presence, healthcare organizations and authorities have access to vast worldwide networks that can disseminate information quickly and prompt large people masses immediately towards public health objectives.31 Utilization of oral health care is restricted due to the lack of global coverage. Social media platforms offer an alternative to traditional communication that reaches underserved communities. WHO and the United States Centers for Disease Control and Prevention (CDC) use social media as many other public health agencies in times of natural disasters and public health crisis.31 Sharing health-related information not only helps to increase knowledge, but can also develop healthy habits and awareness. To give an example, in dentistry social media has beared a part in helping patients deal with dental anxiety and learning treatment options appropriately. Evidence has now shown that social media-based interpositions are linked to healthy behaviors such as quitting tobacco, caring physical activity and avoiding risky sexual behavior.31

Advantages of social media in health services are; accessibility, rapid sharing and response creation, advanced and two-sided communication, reduced consultation time, smoothing of hierarchy, more effective teamwork, the capacity to reach large masses and establish new connections. It also facilitates reach to health information for large groups regardless of age, education or geographic location compared to conventional methods. Like most technologies, social media has its downsides. In the health services; increased workload, unprofessional attitudes, inequality in urgency, feeling of needing to keeping in touch all day long, adversity in collect discussion data, concerns about referring or diagnosing patients, breach of privacy, transformation of the patient-physician relationship from professional to personal are among the disadvantages.

Özdede et al. analyzed the videos of dentistry and the new coronavirus on YouTube and determined that dental YouTube videos from official institutions have a higher information level and quality, also they declared that it would be favorable for experts, universities and other institutions

have upload scientific videos with sufficient duration. The researchers reported that the video contents were insufficient regarding dental emergencies and legal-financial issues, so more videos were needed for these contents. Moreover, it was considered that the health-related videos should be uploaded by subjected to an official control and approval system so it would be advantageous for YouTube to analyze and remove the low-quality videos containing unnecessary/false information and increase the relevance of useful videos when uploading. In this study, it was suggested to users that they should be extremely careful while searching about health issues and that they should choose videos by institutional sources.32 Accordingly, a study presented an analysis of YouTube videos as an informational source for dentists in preventing the COVID-19 outbreak, the credibility of the videos was potentially significant, but shortcomings were found. Researchers noted that only 2 out of 55 videos are in good quality and that there is a huge demand to improve the quality and credibility of information to obtain better results during the pandemic.33

Cabı et al. evaluated the technologies used in distance education in the COVID-19 pandemic, analyzed the findings in more detail and made recommendations for research and practice.34 These recommendations can be listed as follows: Institutional support is important for distance education applications to continue in an effective and beneficial way. It is recommended that digital guides, help videos, in-service trainings and recorded trainings prepared for instructors made available for continuous access. Universities' distance education or continuing education centers should work systematically with the skills and experience to carry out these services for both distance education programs and possible distance education transition scenarios, and efforts should be focused on developing and enriching the distance education process and digital education opportunities. It is suggested that the software and hardware infrastructure of offline platforms should be flexible and scalable, the interface design should be easy to use, and the system should be usable on different platforms such as computers, tablets and mobile phones.

When using online course tools, sharing live lecture recordings with students later, providing a high level of instructor control in meeting settings, having more video participants and institutional support for access to the synchronous tool will be beneficial. It is important that the existing technological possibilities and measurement tools in distance education cannot be completely reliable, so the exams to be held in the electronic environment are supervised and online.34

It is quite clear that social media helps access to information of health. When using social media to share health information, it is considerable to develop messages that are more likely to resonate and elicit

responses from individuals. Messages forwarded to specific population are more influential than general ones because address the specific needs of their receivers. Also, interactive (two-way) communication is more powerful than linear (one-way) communication. Moreover, social media should supplement rather than substitute conventional health services. Social media promotes empowerment of patients by expanding knowledge and putting them in a position to control their own healthcare needs.31,35

It is clear that the use of social media is not a just tendency but a major shift in the communication way of the people today. Multidirectional health services, including social media and other communication forms have been shown to be quite effective. In dentistry, social media can be used to improve oral health, boost public knowledge, simplify research, enhance education and help to battle with public health crisis. Because social media is a relatively new trend in dentistry, more studies are needed to establish its long-term effectiveness and maximize its benefits. In cases of global crisis, content on digital platforms should be analyzed in detail and turned into an advantage in crisis management. In this direction, the most pragmatic use of digital platforms in the current pandemic and possible health crises in the future should be encouraged in order to control the accuracy and reliability of the content in the social media, to compensate for the devastating effects of the pandemic through the creation of new content, to contribute to the literature with the opportunity to conduct wider studies. Thus, it can be ensured that the needs in health-related planning can be quickly understood in pandemic and similar global crisis processes. While social media contributes to the current process, it will provide an advantage for future health crises. The use of social media should be evaluated in the best way to ensure that dental education and treatments are not interrupted and in the fight against all kinds of financial and moral destructive effects of health crisis.

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Chapter 10

INVESTIGATING THE HIDDEN CYCLES EMBEDDED IN THE NEW CASES OF COVID-19

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1. Introduction

Since the beginning of the COVID-19 epidemic, which is one of the biggest pandemics in the history of the world, tourism, economy, etc. heavily damaged areas and continues to do so. Current, almost 250 million people infected leading to 4 million deaths. COVID-19 threats not only the health of the humankind but also our jobs and even our way of life. During the pandemic many organizations and institutions transform their classic office work to remote work. Despite the slowing pace of the epidemic, such changes in business life are still being discussed and may even be applied permanently in some business areas. In this period, the most destructive effect was seen in the education system. In many countries, education was suspended and distance education methods were tried. There are still problems with higher education today. The development of distance education systems has also opened the way for many opportunities. Personal development trainings have been developed with many distance educations programs.

Many businesses had to close during the pandemic. Many people lost their jobs especially in the sectors of service, entertainment, transportation and tourism. Job losses in these sectors and the business closure affect not only people but also the country's economy. For example, tourism is an important input item in the economy of many countries such as Spain, Turkey and Italy. The transportation restriction hurt the tourism revenues of these countries. In countries such as the United States or the United Kingdom, job losses weighed on the economy through unemployment benefits. In addition, infected cases in these countries suffer from health expenditures and inadequate health systems from time-to-time harm the welfare of the people. Industrialized countries such as France, Germany and Japan, on the other hand, caused economic losses due to the restrictions applied especially in the logistics sector due to the pandemic. Curfews implemented in developed and developing countries and the aid provided by governments to their people for these reasons have caused unplanned expenditures and losses in their economies.

For the reasons given in the above Germany, United States of America (USA), France, United Kingdom (UK), Spain, Italy, Japan and Turkey analyzed in the study, while both vaccination activities and measures against the spread continue increasingly.

Currently; Germany is among the successful countries with the measures it took in the first wave of the epidemic, the equipment of the

health system and the planning in the supply of materials. As of October 21, 2022, the vaccination rate is 86%. The USA is one of the countries with the highest number of cases and deaths since the beginning of the epidemic. During this period of just over a year, 844 thousand people lost their lives in the country with a population of 330 million. (The vaccination rate is 85% as of October 21, 2022.) On the one hand, England is one of the countries that experienced the epidemic most severely compared to its population; On the other hand, it is among the countries that start the vaccination campaign the earliest and can vaccinate at the highest rate. In the UK, which started vaccination a month before the EU, there was a serious decrease in the number of cases and deaths, despite the emergence of a faster spreading variant of the coronavirus, together with the vaccination and the strict closure measures. The vaccination rate is 81%. Italy is one of the countries most affected by the epidemic. There is an increase in the epidemic with the effect of new variants in the country. The vaccination rate is 89%. Since the beginning of the epidemic, the total number of deaths has been recorded as 188 thousand. 163 thousand people lost their lives in France with a population of 65 million. In France, which could start vaccination in January together with other EU countries, 82 percent of the population has been vaccinated. The number of people infected with coronavirus in Turkey, whose population is approaching 84 million, is around 91 thousand according to official data. Currently, Sinovac and Pfizer BioNTech vaccines from China and Germany are used in Turkey and the vaccination rate is 84%. In addition to being known as the country where the coronavirus is seen most intensely after Italy in Europe, Spain is also in the third place in the world. The death toll is around 101 thousand. The vaccination rate is 82%.

In April 2020, when the first wave of infections emerged, a decision to shut down was not taken in Japan, but mask obligation and social contact restrictions were imposed, and infection cases were carefully monitored. Thanks to these measures, the spread of the virus was largely prevented. The vaccination rate is 77% and the death toll is around 22 thousand.

The modeling and forecasting of new cases of COVID-19 is very crucial to fight against the pandemic. The decision makers can take preventions and develop models to decrease the spread of the disease by using mathematical and statistical models. In the literature there are many valuable studies which aim to model new cases and new deaths of COVID-19. Here, we try to summarize a few of them to give insights to the reader about the existing and new statistical techniques applied to COVID-19 pandemic.

The time series methodology is proposed by [1] to model new cases of COVID-19 of G8 countries alongside with Turkey. The used models are autoregressive moving average (ARIMA), curve estimation models and Brown/Holt exponential smoothing methods. According to the results of the study ARIMA works better for Turkey, Germany and France while Holt model works better for Canada, Italy, Japan and UK. Logistic, Gompertz and Artificial Neural Network is utilized by [2] to model new cases of Mexico. The findings of the study showed that the proposed models have coefficient of determination very close to 1 while these are capable of predicting the days in which maximum daily cases occur.

The comparison of different lagged autoregressive (AR) model to forecast new cases and deaths of Turkey studied by [3].

The empirical evidences reveled that AR(1) has the highest forecasting power for new cases while AR(2) has the highest performance on forecasting new deaths of Turkey. COVID-19 cases of Nigeria are studied by [4]. The authors compared 36 statistical models alongside with 9 nine curve estimation models. The authors recommended least absolute deviation estimator to forecast new cases.

Confirmed cases and deaths of India is examined by [5] by utilizing fractional-order derivative-based models.

A mathematical model based on differential equations developed by [6] to model official infected cases of India. The proposed model is capable of indicating the increase in the number of asymptomatic patients.

[7] proposed a new mathematical model for the spread of the cases of China. The novelty of the model is that; it takes account the fraction of the detected cases over the real infected cases. The proposed model can be used to determine the number of beds in the hospital for treating COVID-19 cases.

New cases of Germany simulated by [8] by extending the SEIR model. The proposed mathematical model consists of systems of differential equations which considers the age distributions of the cases.

The global data analysis with USA and Germany is done by [9] by employing multiple machine learning algorithms. The results of the study showed that support vector machine has the better forecasting power in detecting the trend in the data.

The global new cases and recovered cases are model by utilizing time series methodologies by [10]. The proposed model is AR based on two-piece scale mixture normal distributions. The empirical results showed that the proposed model has high forecasting power.

The percentage of active cases per population is investigated by [11]. The authors compared many time series methodologies and their findings has mixed results. For example, for the case of USA, Italy and Russia ARIMA worked well but for Turkey the Holt-Winters additive model worked well.

Another time series methodology approach to model infected cases of India is done by [12]. The authors compare ARIMA with non-linear autoregressive neural networks (NAR). The results showed that both ARIMA and NAR models has high goodness of fit.

The number of new cases of Australia, China, France, Italy, Spain, and the USA is modeled by [13]. In the study many deep learning algorithms are employed and the best result is achieved by Variational AutoEncoder model. Another ARIMA based model is proposed by [14]. Their findings showed an increasing trend in the number of new cases of India.

[15] proposed a deep learning algorithm called long-short term memory (LSTM) network to model the new cases of Canada. Also, the transmission rate of Canada is compared with the rates of Italy and USA.

The comparative study of the USA and India is done by [16]. The authors used time series methodologies and the results showed that Convolution LSTM outperforms the other investigated deep learning models.

[17] employed Genetic Evolutionary Programming (GEP) to model daily new cases and deaths of India. The empirical evidences showed that (GEP) is reliable and accurate.

[18] identified the hidden periodic structure of the daily infected cases. Infected case of Turkey is analyzed by using periodogram-based methodology. The results revealed that there are 4, 5 and 62 days cycles in the daily new cases of Turkey.

Other recent time series application on new cases and deaths can be listed as: [19]; [20]; [21]; [22]; [23]; [24]; [25] and [26].

The aim of this study is to investigate the periodic structure hidden in the new cases of COVID-19 of countries which are suffered much in the last 2 years. The hidden cycles represent the waves in the new cases. The length of the waves with the day of the peak can be revealed by this information. The countries selected for this study are: France, Germany, Italy, Japan, Spain, Turkey, UK and USA.

Against to the current literature given in the previous section, the contribution of this study to the literature can be listed as;

- To the best of our knowledge, this is the first study which investigates the hidden periodic structure of the new cases of COVID-19 of the most influenced countries.
- The statistical properties of the examined time series are investigated.
- By making comparisons between the countries examined, the reasons underlying the differences were tried to be revealed.

We believe that these findings provide important information in understanding the behavior and the spread of the COVID-19. The rest of the paper is as follows. Sect. 2 is devoted to the methodological background. Sect. 3 represents the data and analysis. Finally, Sect. 4 concludes this study.

2. Theoretical background

Periodograms are usually used to search the hidden patterns embedded in the underlying time series which is different than the seasonality. The periodicities of a stationary time series can be investigated by employing periodograms. The first assumption in utilizing the periodicities is the stationarity. In the literature there are various unit root test alternatives. The Augmented Dickey Fuller (ADF) test, which is developed based on the asymptotic distribution of the least squares estimators of the parameters, stands out.

Periodograms are derived by using the trigonometric transformations since trigonometric functions have a periodic structure. If we let e_t represent independent and identically distributed random variable with mean 0 and variance σ^2 then for any time series $\{Y_1, Y_2, \dots, Y_n\}$ consider the trigonometric model given in Equation 1.

$$Y_t = \mu + a\cos(w_k t) + b\sin(w_k t) + e_t, t = 1, 2, \dots, n. \quad (1)$$

Here w_k are Fourier frequencies when $w_k = 2\pi k/n$ for $k = 1, 2, \ldots, n$. According to the model given in Equation 1, the least square estimators of the parameters can be obtained as

$$\hat{\mu} = \bar{Y}_n, \qquad a_k = \frac{2}{n}\sum_{t=1}^{n}(Y_t - \bar{Y}_n)\cos(w_k t) \;\; and \;\; b_k = \frac{2}{n}\sum_{t=1}^{n}(Y_n - \bar{Y}_n)\sin(w_k t).$$

Here a_k and b_k are the Fourier coefficients. Moreover, these coefficients are invariant of mean because of the following property of the trigonometric functions

$$\sum_{t=1}^{n}\cos(w_k t) = \sum_{t=1}^{n}\sin(w_k t) = 0.$$

The Fourier coefficients lead us to calculate the ordinate of periodograms when time series has frequency of w_k as

$$I_n = (w_k) = \frac{n}{2}\left(a_k^2 + b_k^2\right). \tag{2}$$

On the other hand, if the time series $\{Y_1, Y_2, ..., Y_n\}$ is stationary and $f(w_k)$ represents the spectral density function then as $n \to \infty$, we have $I_n(w_k)f(w_k) \xrightarrow{D} \chi_2^2$ where $\xrightarrow{D}$ represents convergence in distribution ([27], [28]).

In order to detect the possible periodicities of the time series consider the hypotheses test $H_0: a = b = 0$ for the model given in Equation 1. If the null hypothesis is rejected, then the conclusion of periodicities can be reached. To test the hypothesis standard F test cannot be used because the frequencies, w_k, are unknown [28].

The following test statistics is developed by [28] to handle this situation.

$$V = I_n\left(w_{(1)}\right)\left[\sum_{k=1}^{m} I_n(w_k)\right]^{-1} \tag{3}$$

Where $I_n(w_{(1)})$ represents the highest periodograms value while m represents the greatest integer of $n/2$ that is $m = [n/2]$. If there is no

any periodic component in the data, under the null hypothesis $H_0: a = b = 0$, then for V statistics

$$P(V > c_\alpha = \alpha \cong m(1 - c_\alpha)^{m-1} \tag{4}$$

can be calculated and c_α represents the critical value for a significance level of α [28]. The critical value for any significance level α is calculated as

$$c_\alpha = 1 - \left(\frac{\alpha}{m}\right)^{\frac{1}{m-1}} \tag{5}$$

If $V > c_\alpha$, then the null hypothesis $H_0: a = b = 0$ is rejected and it indicates that the investigated time series contain periodic component. Also, to search further periodic components in the time series define a test statistic as

$$V_i = I_n(w_{(i)}) \left[\sum_{k=1}^{m} I_n(w_k) - \sum_{k=1}^{i-1} I_n(w_k) \right]^{-1} \tag{6}$$

Where V_i is the test statistic for detection of a periodic component and $I_n(w_{(i)})$ for $i = 1, 2, \ldots, n$ are the periodograms values in descending order. If the null hypothesis is rejected, $V_i > c_\alpha$, then it can be concluded that the investigated time series has a periodic component at the corresponding frequency [28].

3. Data and the empirical evidences

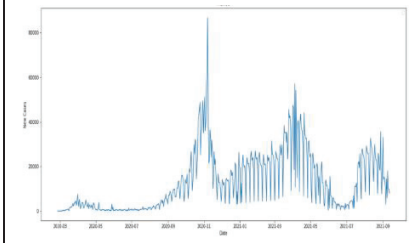
The dataset of this study contains daily new cases of France, Germany, Italy, Japan, Spain, Turkey, UK and USA. The range of the dataset for each country differs. The starting date of the dataset for the countries considered as the data when the first death is encountered.

The summary about the dataset is given in Table 1.

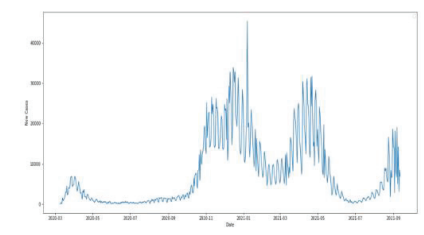
Table 1. Dataset ranges for each country

Country	Dataset Range	Number of Observations
France	2020-02-26 - 2021-09-12	566
Germany	2020-03-09 - 2021-09-12	554
Italy	2020-02-21 - 2021-09-12	571
Japan	2020-03-08 - 2021-09-12	555
Spain	2020-03-03 - 2021-09-12	560
Turkey	2020-03-19 - 2021-09-12	544
United Kingdom	2020-03-06 - 2021-09-12	557
USA	2020-02-29 - 2021-09-12	563

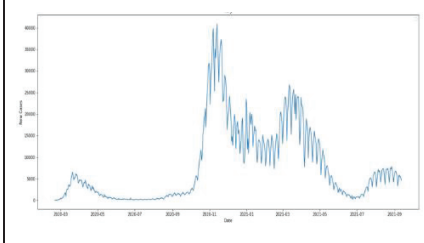
Graph of the number new cases is given in Figure 1 and the graph of number of new deaths is given in Figure 2.



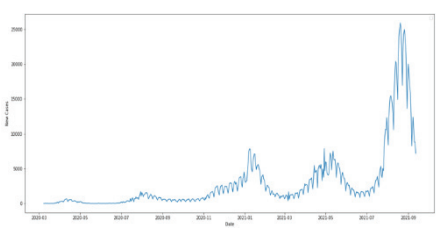
France



Germany



Italy



Japan

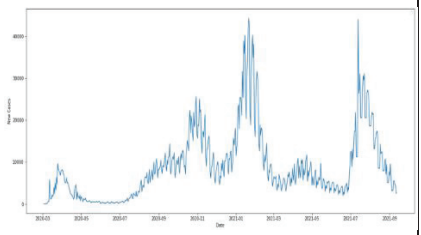
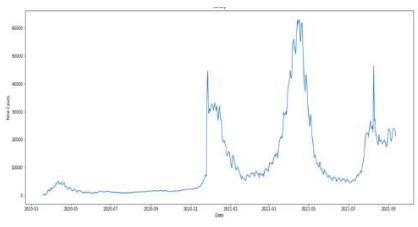
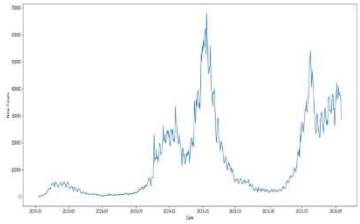
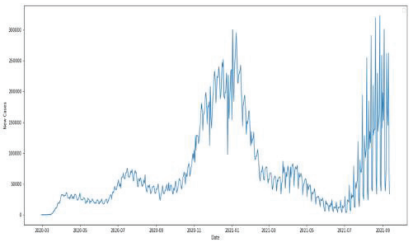
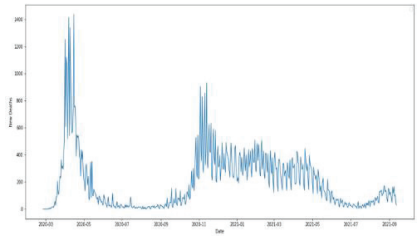
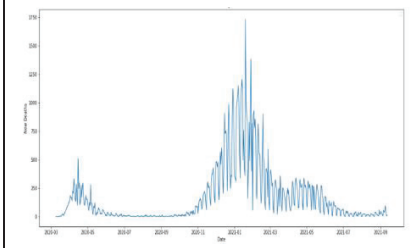
	
Spain	Turkey
	
United Kingdom	USA

Figure 1. Number of new cases by country

	
France	Germany

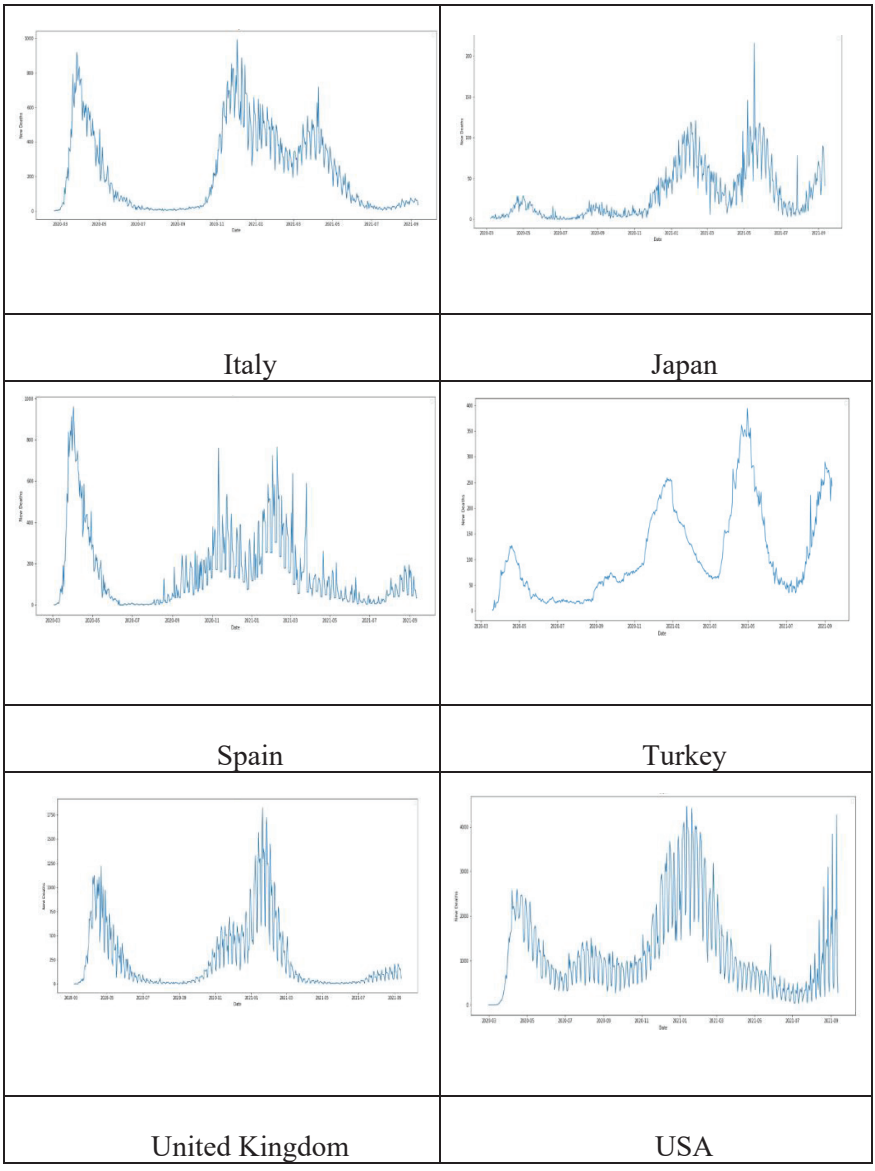


Figure 2. Number of new deaths by country

As Figure 1 and Figure 2 shows that the investigated countries show similarities in number of new cases and new deaths. Peaks in new deaths and cycles are clearly observable. Since neither number of new cases and number of new deaths are non-negative, we transform the data by using

natural logarithm. In the next step stationarity of the time series should be investigated to analysis the periodicities.

We utilized ADF test and the test results are given in Table 2.

Table 2. ADF test result.

Panel A: France			
		t-Statistics	Probability
Augmented Dickey-Fuller test statistics:		-3.256053	0.0175
Test critical values:	1% level	-3.442054	
	5% level	-2.866595	
	10% level	-2.56522	
Panel B: Germany			
Augmented Dickey-Fuller test statistics:		-1.904689	0.3302
Test critical values:	1% level	-3.442367	
	5% level	-2.866733	
	10% level	-2.569596	
Panel C: Italy			
Augmented Dickey-Fuller test statistics:		-3.072432	0.0293
Test critical values:	1% level	-3.441946	
	5% level	-2.866547	
	10% level	-2.569497	
Panel D: Japan			
Augmented Dickey-Fuller test statistics:		-4.108032	0.0010
Test critical values:	1% level	-3.442344	
	5% level	-2.866723	
	10% level	-2.569591	
Panel E: Spain			
Augmented Dickey-Fuller test statistics:		-3.990659	0.0016
Test critical values:	1% level	-3.442142	
	5% level	-2.866634	
	10% level	-2.566543	
Panel F: Turkey			
Augmented Dickey-Fuller test statistics:		-2.929595	0.0427
Test critical values:	1% level	-3.442367	
	5% level	-2.866733	
	10% level	-2.569596	
Panel G: UK			
Augmented Dickey-Fuller test statistics:		-2.479075	0.1212
Test critical values:	1% level	-3.442231	
	5% level	-2.866673	
	10% level	-2.569564	
Panel H: USA			
Augmented Dickey-Fuller test statistics:		-1.966193	0.3020
Test critical values:	1% level	-3.442120	
	5% level	-2.866624	
	10% level	-2.569538	

According to the results given in Table 2; Germany, UK and USA are nonstationary while the rest of the countries' datasets are stationary.

The summary of the unit root test result is given in Table 3.

Table 3. Summary of the ADF test results

Country	Level	First Difference	Conclusion
France	Stationary	Stationary	I(0)
Germany	Nonstationary	Stationary	I(1)
Italy	Stationary	Stationary	I(0)
Japan	Stationary	Stationary	I(0)
Spain	Stationary	Stationary	I(0)
Turkey	Stationary	Stationary	I(0)
UK	Nonstationary	Stationary	I(1)
USA	Nonstationary	Stationary	I(1)

From now on we will work on the level dataset for stationary time series and we use the first differences of the nonstationary time series because periodograms can be investigated only for the stationary time series. Also, it should be noted that the first differences represent the daily changes in the new cases. To investigate the hidden periodicities of the time series V_i statistics are calculated. V_i statistics with their related statistics for I(0) time series are given in Table 4 while I(1) time series are given in Table 5.

Table 4. Five highest period of I(0) time series with their related statistics

i	$I_n(w_{(i)})$	V_i	Period	$c_{0.05}$	Result
Panel A: France					
1	765.5688	0.4946	565.000	0.03018	Significant
2	101.9782	0.1303	282.500		Significant
3	96.5691	0.1419	94.167		Significant
4	74.4083	0.1274	188.333		Significant
5	59.8439	0.1175	70.625		Significant
Total $I_n(w_i)$	1547.95675				
Panel B: Italy					
1	691.4351	0.4409	285.000	0.02999	Significant
2	326.6144	0.3725	570.000		Significant
3	319.3145	0.5804	190.000		Significant
4	27.3066	0.1183	71.25		Significant
5	15.9839	0.0785	57.000		Significant
Total$I_n(w_i)$	1568.20334				
Panel C: Spain					
1	354.3102	0.3799	559.000	0.0346	Significant
2	224.0090	0.3875	279.500		Significant
3	99.7061	0.2815	93.167		Significant
4	50.5575	0.1987	186.333		Significant
5	27.7823	0.1363	111.800		Significant
Total $I_n(w_i)$	932.458546				
Panel D: Japan					
1	608.766	0.4101	554.000	0.03075	Significant
2	204.430	0.2335	277.000		Significant

3	125.894	0.1876	184.667		Significant
4	121.872	0.2235	92.333		Significant
5	105.635	0.2495	138.500		Significant
Total $I_n(w_i)$	1484.36946				
Panel E: Turkey					
1	560.5475	0.6331	543.00	0.03124	Significant
2	100.8175	0.3104	135.75		Significant
3	68.5722	0.3062	108.60		Significant
4	26.2767	0.1691	271.50		Significant
5	19.6677	0.1532	60.333		Significant
Total $I_n(w_i)$	885.335063				

Table 5. Five highest period of I(1) time series with their related statistics

Panel A: Germany					
i	$I_n(w_{(i)})$	V_i	Period	$c_{0.05}$	Result
1	1073276321	0.11142	6.975	0.03124	Significant
2	560105369	0.06543	7.064		Significant
3	290562197	0.03632	2.296		Significant
4	195489218	0.02536	6.639		Insignificant
5	194996727	0.02595	6.888		Insignificant
Total $I_n(w_i)$	9633043866				
Panel B: UK					
1	272228908	0.07073	7.025	0.03074	Significant
2	121383572	0.03393	7.208		Significant
3	106480718	0.03082	6.938		Insignificant
4	91328378	0.02727	2.211		Insignificant
5	90704572	0.02785	2.194		Insignificant
Total $I_n(w_i)$	3848756140				
Panel C: USA					
1	61168774080	0.06354	3.484	0.03239	Significant
2	48383648924	0.05367	3.528		Significant
3	47170577629	0.05529	3.506		Significant
4	42144867541	0.05229	2.328		Significant
5	31786323455	0.04162	3.551		Significant
Total $I_n(w_i)$	9.62615E11				

According to the results of Table 4 new cases of France have periods of 70 and 94. The periods of 565, 283 and 188 corresponds to the whole the half and the one third of the dataset, respectively. For that reason, they are ignored. Italy's new cases have periods of 57 and 71 while periods of 190, 285 and 570 are ignored. The new cases of Spain have 93 and 112 periods while 186, 278 and 559 periods are ignored. In the case of Japan new cases have periods of 92 and 139 while 185, 277 and 554 periods are

ignored since they represent the one third, the half and the whole of the dataset. Lastly, new cases of Turkey have periods of 60 and 109 while the periods which indicate the half of the data, the one third and the whole period of the data are ignored.

The summary of the periods of the new cases are given in Table 6.

Table 6. Summary of the periods of the new cases

Country	Periods
France	70, 94
Italy	50, 71
Spain	93, 112
Japan	92, 135
Turkey	60, 109

The Table 5 indicates that change in the new cases of Germany has periods of 2 and 7 days while UK has 7 days and finally USA has 2- and 3-day periods.

4. Conclusion

The COVID-19 measures are implemented into five categories such as social distancing, movement restrictions, public health measures, social and economic measures, and lockdowns applied by governments worldwide in the coronavirus pandemic as in European countries. Although new COVID-19 cases differ according to the restrictions applied in each country, waves in the number of cases have been observed to have similar patterns. Hidden periods in France, Italy, Spain, Japan and Turkey vary between 50-93 days. This shows that measures are being reviewed to prevent the epidemic in approximately 2-3 months. The operation of COVID-19 and its variants is in two-month cycles, but the cycle is said a product of “human nature” rather than “mother nature”. The decrease in COVID-19 cases can cause to become more careless for the protection from virus and loosening of governments restrictions. This situation can cause that an increase in the number of cases reaches a new peak for the following period over 2-3 months. Spain and Italy experienced the most effective epidemic in Europe. People were under lockdown for three months and many young people stayed in their family homes. Especially in Spain and Turkey, unsurprisingly street parties and nightlife can be signaled as the biggest catalysts of new outbreaks along with family gatherings.

The hidden periods of change in new cases of Germany, the UK, and the USA vary between 2-7 days. It can be caused from the incubation period of the COVID-19 and the social gatherings of weekends. 4 day

period represents the incubation while 5 day period represents the weekend affect. Also, it should be noted that less mask-wearing, relaxed many restrictions, protection against catching the virus wanes are reasons to increase the number of new cases in the UK. The increase in new cases in such a short period may be due to the easing of the measures taken against the pandemic on weekends.

Although there is a high risk of transmission in the community, it continues to maintain face-to-face education in a fully open/partially open status using routine rapid antigen tests in schools in Spain, France, Germany, the UK, and the USA. Therefore, the implementation of the decision to continue fully open / partially open education in schools depending on the spread of the virus and increase to cases is one of the most important factors causing the fluctuation in the number of cases.

The high numbers of new cases remain attributable to the spread of the highly infectious delta variant, which usurped previous variants more infectious than the original strain of Covid-19.

After all the vaccination period progresses rapidly, the fluctuations in the virus still continue the virus effect. This brings the governments to the agenda to take effective measures. During the pandemic, the factors, the effectiveness of vaccines over time, infection prevention policies, different variants of coronavirus, human behavior, and undeveloped immunity problems, have had an impact on whether new cases are increasing or declining.

Coronavirus tends to be more spread in places where people live or work closely together like multigenerational households, long-term-care facilities, prisons, and some types of businesses. The COVID-19 outbreak, which ranks first in the list of the largest epidemics the world has been exposed to; it has been an experience that requires many lessons to be learned with all the measures taken or not taken before, during and after. What has been experienced administratively since the beginning of the epidemic is the biggest indicator of how ready we are for such crises. Another problem encountered in the epidemic is that there are problems in the health systems of countries, such disruptions will of course pave the way for better health systems. As the old Turkish proverb says, "one misfortune is better than a thousand advice".

In the ongoing pandemic process, countries continue to take measures and try to increase vaccination rates. Although the measures taken show slight differences, in general, it is seen that the some of the measures will

continue until the end of the pandemic. Also, the global data trends show that the pandemic isn't over yet.

While people's restrictions are starting to be relaxed in many countries to control the epidemic, the epidemic continues to affect especially African countries where adequate vaccination is not done and access to vaccines is limited. Therefore, the measures taken against the epidemic will be successful when all countries reach vaccination at similar rates. With the spread of new mutants, it is very important to constantly maintain different measures and courses of action for public health.

Although the pandemic process is not over, restrictions are being lifted in many countries. One of the most important lessons to be learned in this process is to strictly comply with the restrictions. The ongoing process should also be evaluated well against different epidemics that may occur in the future.

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Chapter 11

ASSESSMENT OF POTENTIAL RISK FACTORS FOR IMMEDIATE IMPLANT PLACEMENT IN THE POSTERIOR MANDIBLE

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INTRODUCTION

Functional, aesthetic and emotional discomforts are generally observed in edentulous individuals. Tooth-supported fixed and partial dentures, dental implant-supported dentures are possible solutions to these problems (Asawa et al., 2015).

Rehabilitation of edentulous areas with dental implants is one of the important developments in dentistry (Alghamdi, 2018) and has become an integral part of patients' aesthetic and functionally satisfactory goals (Yoon et al., 2017). Moreover, the use of dental implants for rehabilitation provides additional advantages over tooth-supported fixed dental prostheses, such as not restoring adjacent teeth and preserving alveolar bone (Garber, 1996; Reikie, 1993).

Expecting long-term success from a dental implant-supported restoration is dependent on successful osseointegration (Albrektsson et al., 1981), and adequate bone quantity and quality are important factors to ensure proper implant placement (Güncü et al., 2011). With the conventional protocol, it is stated that a period ranging from about 3 to 8 months (according to different jaws) should be waited without any loading after the implants are placed in order to achieve the desired osseointegration (Branemark et al., 1977). This recommendation was based on the belief that in order to achieve the goal of successful osseointegration, the soft and hard tissues must be completely healed after extraction (Adell et al., 1981). However, as a result of tooth extraction, dimensional changes characterized by resorption of alveolar bone and, accordingly, retraction of soft tissues can be observed (Abd-Elrahman et al., 2020; Siormpas et al., 2018). Moreover, for the implementation of the conventional protocol, there is a need for a second surgical procedure to place the implants following the extraction site healing period, and even subsequently a third surgical procedure to expose the implants for restoration of the respective implants. In this respect, this protocol technically guides patients to more than one surgical intervention (Branemark et al., 1977; Chen et al., 2004).

Thanks to modern and evolving implantology, it is now possible to replace the conventional approach introduced many years ago (Mello et al., 2017). Immediate implant (IMI) placement, which is a very remarkable and predictable protocol (Ebenezer et al., 2015; Swathi, 2016; Waasdorp, 2018), refers to implantation into the extraction socket immediately after extraction (Koh et al., 2010). Compared to implants placed in healed ridges, IMI placement in extraction sockets is a predictable treatment modality with documented comparable survival rates (Chen et al., 2004; Schwartz-Arad & Chaushu, 1997; Wagenberg & Froum, 2006). When indicated, placing IMI in extraction sockets offers many advantages (Al-

Sawai & Labib, 2016; Esposito et al., 2006; Sennerby & Gottlow, 2008): fewer surgical procedures, shorter treatment time, (Becker et al., 1998; Lazzara, 1989; Schwartz-Arad & Chaushu, 1997) and also providing psychological benefits to patients (Koh et al., 2010).

In the literature, extraction of mandibular molar teeth is a common phenomenon for various reasons (endodontic failure, caries, vertical root fracture, etc.), and in such a case immediate implantation can be considered (Hamouda et al., 2015). It has been reported that immediate implantation is a predictable approach, especially in posterior regions where aesthetics is not a priority (Lang et al., 2012; Strub et al., 2012), and IMI placement in molar extraction regions is a valid approach with high success and survival rates (Hayacibara et al., 2013; Ketabi et al, 2016; Ragucci et al., 2020).

While primary implant stability is recognized as a fundamental requirement for successful osseointegration (Zhou et al., 2008), it is clear that anatomical morphology is an important factor influencing primary stability and osseointegration (Meijer & Raghoebar, 2020). In particular, immediately placed implants are more stable in the anterior region than in the posterior region (Mesa et al., 2008). Anatomical structures such as the inferior alveolar nerve, submandibular fossa, and interradicular septum, which may exhibit limiting features, may prevent the primary stability of the implant during immediate implantation (Froum et al., 2011). The posterior mandible therefore presents a unique challenge for the desired level of IMI placement (Greenstein et al., 2008).

Lingual concavities

The submandibular fossa is a concavity in the mandibular molar region, lingual to the mandibular body, inferior to the mylohyoid line, and also where the submandibular gland is located, and there are many important anatomical structures around it. Therefore, it is important to fully understand the surgical anatomy in the area to prevent complications related to this area from occurring (Harazono, 2019).

The source of the concavity in the lingual part of the mandible is the compressive forces of the submandibular and sublingual salivary glands on the mandibular cortex (Philipsen et al., 2002). Various degrees of lingual concavities are frequently observed in the posterior mandible (Kamburoglu et al., 2015), and lingual undercuts are more common, especially in the edentulous mandible (Nickenig et al., 2015). In addition, it was observed that the age of the patient, the presence of teeth and the location of the concavity had an effect on the dimensions of the aforementioned concavities. (Kamburoglu et al., 2015). The presence of deep lingual concavities potentially leads to an increased risk of lingual cortical perforation

during implantation. During placement of an implant that does not have an appropriate angle and position, the bone boundary may be violated (Chan et al., 2011a & 2011b; Parnia et al., 2010) and the surrounding vital structures may be damaged (Froum et al., 2011; Quirynen et al., 2003). A perforation above the mylohyoid ridge may cause damage to the lingual nerve, and a perforation below the mylohyoid ridge may cause damage to the inferior alveolar nerve (Chan et al., 2011b). Moreover, severe hemorrhage with life-threatening consequences and subsequent hematoma can be observed due to upper airway obstruction as a result of damage to the submandibular gland fossa through perforation of the lingual plate in the surgical procedure (Rajput et al., 2018).

Preoperative evaluation of morphology is an important requirement to prevent the aforementioned complications. The lingual concavities should be carefully palpated before osteotomy is performed (Bayrak et al., 2018). However, while deep and risky mandibular lingual concavities cannot be visualized on two-dimensional images (Bodart et al., 2020), cross-sectional analysis of three-dimensional radiographs provides an opportunity to identify features of a lingual concavity and prevent the complication of perforation (Chan et al., 2011a).

Inferior alveolar nerve

The distance between the root apex and furcations of mandibular first molars and the alveolar nerve determines the dimensions of the residual alveolar bone for immediate implantation (Padhye et al., 2020). In addition, primary stability is considered one of the most important factors for implant stability and is expressed as biomechanical stability after placement of a dental implant (Gomez-Polo et al., 2016; Papadpyridakos et al., 2012). Anatomy of the posterior mandible, including location variability in the submandibular fossa and inferior alveolar canal, may lead to complications such as lingual plate perforation and inferior alveolar nerve injury when primary stability is desired thanks to the amount of natural bone apical to the extraction socket (Greenstein et al., 2008).

In some cases, bone may atrophy to the extent that it causes damage to the inferior alveolar nerve when desirable long implants are placed (Lorean et al., 2013), and thus posterior or lateral mandibular atrophies are a common problem for patients requiring rehabilitation with dental implants (Fernandez-Diaz & Naval-Gias, 2013). Due to the low residual bone quality and quantity, especially in individuals with long-term edentulism, surgical and anatomical difficulties are encountered in the rehabilitation attempt with implants in the mandibular posterior regions (Lorean et al., 2013). As a result of these difficulties, injury to the inferior alveolar nerve or its mental branch may be caused during implantation. This may

result in a temporary or permanent change in sensation in the area concerned. Considering that the mental region is important in terms of very important functions such as speaking, chewing and facial expressions, an injury that may occur may cause significant discomfort in the patient (Givol et al., 2013). In order to prevent damage to the inferior alveolar nerve, various therapeutic approaches have been developed to cope with the deficiency in bone height in cases where severe atrophy is observed. These approaches include the use of short implants, bone augmentation, and more complex and detailed imaging studies that allow nerve lateralization (Chrcanovic & Custo´dio, 2009; de Vicente et al., 2016; Jensen & Nock, 1987; Levin et al., 2007). In addition, pre-procedure use of an imaging that allows three-dimensional analysis such as CBCT will make a great contribution to determining the location of the inferior alveolar canal and deciding on an appropriate treatment approach.

Interradicular septum

Failure to achieve primary stability during the surgical procedure is one of the most important complications and may mean failure. Primary implant stability, overexpansion of the implant bed, poor bone quality, or improper bone-to-implant relationships are compromised during immediate implantation (Lamas Pelayo et al., 2008). Since the primary stability of an immediately placed dental implant depends on the area of the implants to be placed, implants placed immediately, especially in the anterior or premolar region, offer more desirable primary stability due to the smaller socket size and less load these implants are subjected to (Atieh et al., 2013). In the molar region, this is controversial because the socket size is large compared to the existing implant diameter (Smith et al., 2019). Primary stability in IMI placement is provided by the interradicular septum and/or the apical natural bone. The risk of immediate implantation failure will be higher if factors such as the interradicular septum, socket morphology, and the space between the implant and the bone are not taken into account (Sayed et al., 2021). Therefore, large extraction sockets, poor bone quality, and low amount of apical native bone (due to restriction of the alveolar inferior nerve) often present clinicians with difficulties in placing IMI in molar extraction sockets (Atieh et al., 2010).

The advantage of using the extraction socket as a guide in immediate implantation has been reported (Fugazzotto, 2002a & 2002b; Garber et al., 2001; Maksoud, 2001). However, immediate implantation into extraction sites of multirooted teeth often poses a dilemma as to how to place them in the ideal position without sacrificing primary stability. Mainly, the morphology of the encountered extraction socket leads to this problem (Rodriguez-Tizcareño & Bravo-Flores, 2009). In studies, the interradicular septum is generally emphasized as the best implant position in terms of

prosthetics (Agostineli et al., 2018; Fugazzotto & Hains, 2013; Sayed et al., 2021; Valenzuela et al., 2018).

The interradicular septum is the bone structure in the socket that separates the tooth roots from the furcation to the apical tip of the roots (Stanley, 2010) and plays an important role in primary stability when there is no natural bone available at the apical part of the roots. However, although the interradicular septum represents the ideal site for immediate implantation for the posterior region (Sayed et al., 2021), the inadequate interradicular septum dimensions observed in some cases may compromise IMI placement procedures (Liechtung, 2012). Therefore, precise knowledge of posterior region anatomical structures, due to the complexity of their anatomy, is of great benefit when tooth extraction and immediate implantation are planned. Here, the surgeon has to evaluate the interradicular septum and its dimensions and the potential presence of apical native bone to achieve primary stabilization of the implant (Hwang & Park, 2008; Esposito et al., 2007). CBCT is of great benefit in obtaining detailed morphological features of the interradicular septum in the mandibular posterior region (Pavlovic & Petrovic, 2022).

CONCLUSION

It is well known that after tooth extraction, there is a decrease in the vertical and horizontal directions of the alveolar crest (Lekovic et al., 1998; Schropp et al., 2003). With this loss of the alveolar crest, the chance of rehabilitation with the implant is reduced, so it has been suggested that IMI placement may counteract this resorption process (Botticelli et al, 2004; Degidi et al., 2007; Oghli & Steveling, 2010). Immediate implantation and prosthetic approach together with atraumatic extraction have been suggested as alternative treatments to preserve tissue volume and reduce treatment time and cost (Grecchi et al., 2009; Mahesh et al., 2012). Proper positioning of implants is important to success in prosthetic restoration (Kopp et al., 2003). However, immediate implantation should be considered a sensitive procedure; therefore, it should always be kept in mind that certain risks and complications may be encountered (Chan et al., 2011a; Froum et al., 2011; Juodzbalys et al., 2013; Waasdorp et al., 2010).

The morphology of the molar extraction socket determines the desired primary stability in immediate implantation (Sayed et al., 2021). Certain difficulties may be encountered in implant planning in the mandibular posterior region: proximity of the inferior alveolar canal, presence of lingual concavities, lack or insufficiency of the interradicular septum, etc. (Rajput et al., 2018). Therefore, preoperative imaging is imperative to ensure success in IMI placement. CBCT scanning is a useful diagnostic

tool in appropriate treatment planning as it can provide important information about anatomical structures and morphological variations in the mandibular posterior regions (Yoon et al., 2017). There is also a wealth of evidence pointing to the reliability and accuracy of CBCT in assessing bone morphology and dimensions (Swasty et al., 2009; Veyre-Goulet et al., 2008). Having knowledge about anatomical structures and variations reduces the risk of complications such as neurosensory discomfort, lingual plate perforation, etc. and thus dental implant failure (Güncü et al., 2011; Misch & Crawford, 1990).

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Chapter 12

HYPOTHYROIDISM IN DOGS

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1. INTRODUCTION

Thyroid hormones are involved in a wide variety of metabolic processes, and low thyroid hormone levels cause a variety of clinical and laboratory abnormalities that suggest hypothyroidism. Hypothyroidism is a common endocrine disease of dogs and generally occurs when adequate amounts of thyroxine (T4) and triiodothyronine (T3) hormones cannot be produced. Since this endocrine disorder requires lifelong treatment, it is very important to make a definitive diagnosis before starting treatment. For this reason, in this review, the thyroid gland, its function, the development mechanism of hypothyroidism, clinical findings, diagnosis, treatment and management of the disease are discussed.

2. THYROID GLAND AND FUNCTION

Thyroid gland is the one of the most important gland in the body. It is found in all vertebrates and is usually two-lobed in mammals. It is located in the upper 1/3 of the neck region, adjacent to the trachea and just behind the larynx. Its shape similars a butterfly or tied tie knot and is about the size of a bean. A disorder that can occur at any point in the hypothalamus-pituitary-thyroid axis causes thyroid hormone deficiency (hypothyroidism) or excess (hyperthyroidism). In 95% of clinically observed hypothyroidism, the problem is the thyroid gland itself (primary hypothyroidism). The two most common causes of primary hypothyroidism are lymphocytic thyroiditis (autoimmune thyroiditis) and atrophy of the thyroid gland. Less common causes of hypothyroidism are iatrogenic conditions, tumors developing in the thyroid gland, and congenital (congenital) hypothyroidism (Feldman and Nelson 2003, Başoğlu and Sevinç 2004, Dodds and Laverdure 2011).

The thyroid gland is responsible for take iodine from food and the thyroid uses iodine in the production of the hormones T4 (tetraiodothyronine, thyroxine) and T3 (triiodothyronine). Although 80-90% of the produced hormone in the thyroid gland is T4, only 10-20% is T3. T3 hormone is 3-10 times more active than T4 hormone. Therefore, T4 hormone must be converted to T3 hormone in order for cells and tissues in the body to benefit from thyroid hormone. Approximately 80% of this transformation occurs in the liver and the remaining 20% occurs in the skin, kidneys, muscles, spleen and central nervous system (Dodds and Laverdure 2011, Chastain and Panciera 1995).

Synthesized hormones from the thyroid gland are tetraiodothyronine = thyroxine (T4), triiodotrinonin (T3) and calcitonin. T4 and T3 hormones are made of amino acids, and the hormone calcitonin is polypeptide. High percentage of the iodine in the body is found in the thyroid gland. iodide is

also found in the thyroid gland, gastric mucosa, salivary glands, placenta, ciliary body of the eye, choroid plexus and mammary glands. If the substances that play a role in the production and release of thyroid hormone and this pathway are briefly defined;

1. Iodide is taken up by the Sodium Iodide Simporter (NIS) from the basement membrane of the thyroid follicle.

2. It is transported apically from the basement membrane and passed into the lumen of the follicle via Pendrin.

3. Tyrosine Peroxidase (TPO) enzyme; converts iodide to iodine. Iodine binds to tyrosine in thyroglobulin via the enzyme iodinase. The binding of iodine to tyrosine is called organification. TPO is located on the membrane at the apical part of the cell.

4. Mono-iodotyrosine (MIT) is formed if one iodine is bounded to tyrosine, and Di-iodotyrosine (DIT) is formed if two iodines are bounded. An MIT and a DIT consolidate to form T3, and two DITs combine to form T4. (MIT + DIT =T3, DIT + DIT = T4)

5. This structure is taken into the cell through the megalin in the apical membrane of the cell.

6. Proteases digest thyroglobulin. Thus, MIT, DIT, T3 and T4 bound to thyroglobulin become free.

7. T3 and T4 pass into the blood. MIT and DIT are separated from its iodine by the deiodinase enzyme and these iodines are reused in the synthesis of thyroid hormone.

Since the liver plays a very important role in the conversion of the T4 hormone to the T3 hormone, the de-iodination process may be impaired in acute or chronic liver diseases and the T4 hormone in the blood cannot be effectively refer to the tissues as T3. In situation, animals with normal blood thyroid levels show clinical findings of hypothyroidism. Thyroid hormones control the functions of organs and tissues (protein, carbohydrate and fat synthesis, enzymes, vitamins and minerals) by increasing basal metabolism, regulating body temperature, regulating heart and respiratory rate. It also takes part in the production of the hormone calcitonin, which plays a important role in the regulation of blood calcium level and metabolism in the body. It also plays an significant role in the development of the brain. It takes part in uterine functions and its preparation for reproduction (Başoğlu and Sevins 2004, Dodds and Laverdure 2011, Merck, 2013).

2.1. HYPOTHALAMUS-PITUITARY-THYROID GLAND AXIS

The action of the thyroid gland to produce hormones is controlled by the pituitary and hypothalamus. Both of these glands are localized in the head region. These glands, together with the thyroid, form the hypothalamic-pituitary-thyroid axis. The pituitary gland produces and secretes the hormone called thyroid stimulating hormone (TSH). This hormone is also expressed as thyrotropin (Başoğlu and Sevinç 2004). TRH released from the hypothalamus (TRH; circadian and pulsatile release); stimulates the release of TSH from the pituitary. TSH, on the other hand, stimulates the thyroid gland to release T4 and T3. Increasing TSH level inhibits TRH release with negative feedback. Increasing T4 and T3 levels inhibit both TRH and TSH release. The amount of released thyrotropin from the pituitary gland depends on the amount of free T3 and free T4 coming from the blood to the pituitary. The more free T4 and T3 stimulate, the more TSH is produced from the pituitary. Therefore, it acts as a regulator sensor of the body and thyroid hormone is produced according to the body's needs. If everything is normal, TSH secretion allows the level of T4 and T3 in the circulation of the thyroid gland to remain constant (Merck 2013, Dodds and Laverdure 2011).

The key difference between dogs and humans regarding the TSH hormone released from the pituitary gland is that in humans it controls about 95% of thyroid hormone regulation, while in dogs it controls about 70%. The remainder of thyroid hormone regulation in dogs is controlled by somatotropin, also known as growth hormone. Growth hormone is produced, stored and secreted by the pituitary gland like TSH (Dodds and Laverdure 2011). Thyrotropin releasing hormone (TRH), produced and secreted by the hypothalamus, regulates TSH production from the pituitary gland. For example, TRH secretion increases in cold weather. In this case, more TSH is secreted from the pituitary gland and the thyroid hormone activity, which is responsible for accelerating metabolism to regulate (increase) body temperature, increases. (Dodds and Laverdure 2011).

3. FINDINGS OF THYROID DISEASES IN DOGS

It's a confusing situation for dogs to get thyroid disease too early. Until the mid-1990s, the consensus among veterinarians was that hypothyroidism was not seen in younger dogs than 5-7 years of age. However, today, the situation related to this disease can be diagnosed regularly in dogs from puberty (10, 12 or 14 months old) to 2.5 years old (Dodds and Laverdure 2011). Since thyroid hormones affect almost all systems of the body, the spectrum of possible clinical manifestations is quite wide.

3.1. Changes in cellular metabolism: Mental stagnation, weight gain. cold intolerance, lethargy, exercise intolerance, altered mood (sometimes aggressive, sometimes calm), neurological findings (polyneuropathy, growth retardation, seizures), Neurological findings (polyneuropathy, growth retardation, seizures), Chronic infections, Neurological findings (polyneuropathy, growth retardation, seizures). (Scott-Moncrieff 2007, Jaiswal et al. 2018)

3.2. Neuromuscular problems:, Tragic facial expression, joint pain, muscular stiffness, megaesophagus, laryngeal paralysis, facial paralysis, muscle weakness, head tilt, , sagging eyelids, Urinary incontinence. (Jaiswal et al. 2018)

3.3. Dermatological problems: Dry, scaly skin, bilaterally symmetrical hair loss, chronic skin odor, rat or mouse tail, seborrhea with greasy or dry skin, hyperpigmentation. (Jaiswal et al. 2018)

3.4. Reproductive disorders: Infertility, prolonged oestrus intervals, lack of libido, testicular atrophy, azoospermia. (Jaiswal et al. 2018)

3.5. Cardiac abnormalities: Bradycardia, low QRS voltages, inverted T waves, weak apex beat, cardiac arrhythmia, cardiomyopathy. (Scott-Moncrieff 2007)

3.6. Gastrointestinal disorders: Diarrhea, constipation, vomiting. (Jaiswal et al. 2018)

3.7. Ocular disorders: Corneal lipid deposition, uveitis, keratoconjunctivitis sicca or dry eye, corneal ulceration, infections of the eyelid glands (Meibomian gland), Horner's syndrom. (Jaiswal et al. 2018)

3.8. Associated Other Disorders: Glycosuria, loss of sense of smell, loss of taste, other endocrinopathies (adrenal, parathyroid and pancreatic) that may cause chronic active hepatitis. (Dodds and Laverdure 2011).

4. THE MOST COMMON SYMPTOMS OF HYPOTHYROIDISM IN HUMANS AND DOGS

1. Weight gain despite restriction of energy intake; Metabolism slows down in patients with hypothyroidism and dogs become obese. Obesity and insulin resistance may develop in patients. With the slowing of metabolism, heat production also decreases and patients are usually hypothermic. Due to hypothermia, patients seek warm places and are intolerant to cold (Schoeman 2011, Dodds and Laverdure 2011).

2. Dermatological (Hair and hair cover) changes; Dermatological changes take an important place among the most striking findings in patients with hypothyroidism. The skin in these patients is usually dry and

dull. However, sometimes oily appearance, flaking of the skin and bad odor can also be observed. Delay in wound healing associated with insulin resistance may be observed (Schoeman 2011). Bilateral, symmetrical and nonpururitic truncal allopia is an important finding in patients with hypothyroidism. In patients, “rat tail” is formed by shedding of hair in the tail area. Frequently, the patient's owners complain that the hairs break easily and that the regrowth is slow (Kemppainen and Behrend 2001). In two-thirds of dogs with hypothyroidism, hair loss is seen in the lower abdomen, lateral chest region, caudal aspect of the hip region, entire tail, under the neck, and nose. Hyperpigmentation can be seen in the areas where the hairs are shed. Itching and recurrent otitis externa can be observed in cases with secondary pyoderma (Merck 2013)

3. Fatigue, depression and anxiety; Mental state is the most affected part due to hypoxia. Lethargy with hypothyroidism is depressed and lethargic. Symptoms develop slowly and are often overlooked by their owners. However, careful patient owners notice the mental change easily and apply to the veterinarian (Schoeman 2011). Adrenal fatigue or burnout syndrome (also known as adrenal fatigue syndrome) occurs when the adrenal glands produce excess cortisol in response to stress. Excessive production of cortisol causes the glands to not function properly. Adrenal burnout is typically a temporary condition and causes disruption of the activity of essential glands such as the thyroid gland. Thyroid function returns to normal once the condition that caused adrenal depletion is resolved in the first place. Meanwhile, nutritional supplements and thyroid support are made and may be beneficial. Human physicians and veterinarians resist thyroid treatment in adrenal fatigue syndrome. Because they do not treat thyroid disorder and temporarily treat adrenal malfunction syndrome. In this case, nutritional thyroid support or thyroid hormone therapy provides significant improvement in the treatment of adrenal fatigue syndrome (Dodds and Laverdure 2011).

4. Mixedema and coma; Thickening of the dermis is mostly seen as a result of accumulation of glycosaminoglycan (hyaluronic acid) in the skin. Thickening of the skin is usually on the face and forehead. Thickening of the skin on the face and drooping of the eyelids cause the appearance of tragic facial expression in dogs (Merck 2013). As the disease progresses, hypoventilation, bradycardia, and hypothermia occur in a coma state. Coma is a very rare finding (Schoeman 2011, Merck 2013).

5. Gastrointestinal problems; Diarrhea or constipation, rarely megaesophagus and megacolon may be seen due to decreased electrical activity in gastrointestinal smooth muscles (Schoeman 2011, Dodds and Laverdure 2011).

6. Menstrual irregularity and fertility problems; Reproductive problems are one of the first signs in dogs with hypothyroidism. While females have menstrual irregularity, persistent anestrus, and galactorrhea (a milky fluid in the breast, except during pregnancy and lactation), males experience testicular atrophy, gynecomastia (feminine breast enlargement in males), loss of libido, azoospermia (lack of sperm in semen), and orchitis (Schoeman 2011, Dodds and Laverdure 2011)

7. Neurological Symptoms, Muscle and Joint pains; General weakness is among the most common problems in dogs with hypothyroidism. In addition, patients may experience decreased deep sleep, tremor, and myopathy (Tilley and Smith 2008).

5. CLINICAL PATHOLOGICAL FINDINGS

About 30% of adult hypothyroid dogs have mild non-regenerative anemia. Non-regenerative anemia has been reported in congenital hypothyroidism in previously reported case reports. In a significant proportion of cases of subsequent hypothyroidism, serum biochemistry typically reveals fasting hypercholesterolemia and hypertriglyceridemia (Dixon et al. 1999). Thyroid hormones affect lipid metabolism very closely. Diminished degradation of lipids are observed in hypothyroidism (Scott- Moncrieff and Guptill-Yoran 2005). This causes lipid accumulation in the plasma. These changes are not specific to hypothyroidism. However, in cases with appropriate clinical findings, it provides supporting evidence. Less commonly reported abnormalities include ALP, ALT, and CK enzyme activities in serum, and an increase in blood BUN concentration. These changes are highly inconsistent and their association with hypothyroidism cannot be explained. It is stated that in congenital hypothyroidism in humans, hypercalcemia occurs secondary to decreased renal clearance of calcium and increased gastrointestinal absorption. However, this has not been confirmed in dogs and cats (Bojanic et al. 2011).

6. DIAGNOSIS

6.1 Diagnostic Tests and Interpretation

6.1.1.Total T4: This test is the measurement of the total amount of T4 (thyroxine) in the bloodstream. Total T4 contains both bound and free T4. More than 99% of the T4 hormone is "bounded". This means it is attached to proteins in the blood and can never reach the tissues. That's why only the T4 result often causes errors. This parameter can change the amount bound to proteins in the bloodstream by drug or anything else. However, the T4 concentration is still the most popular and widely used test for detecting thyroid disorders in dogs for the first time. In canine thyroid

disorders, the T4 concentration alone is not a definitive indicator and is often affected by moderate or severe non-thyroidal diseases (non-thyroidal diseases) or some drug (phenobarbital, corticosteroids, and sulfanamides) (Scott-Moncrieff 2007, Bruyette 2020). Total T3, total T4, free T3, free T4 and TgAA (thyroglobulin autoantibodies) values should be checked to determine thyroid disorder or to have an idea about the thyroid panel of animals (Scott-Moncrieff 2007, Daminet 2010, Dodds and Laverdure 2011).

6.1.2. FreeT4: The serum free T4 concentration is the very small (< 0.1%) non-protein bound fraction of the thyroxine hormone (Bruyette 2020). The free T4 fraction is the biologically active fraction. Free T4 circulates throughout the body through the bloodstream and as a sensor of the pituitary gland determines whether to produce more Throid Stimulating Hormone (TSH). Although both the free and bound part of the T4 hormone are in circulation, the pituitary gland recognizes the free T4 hormone only. Since the free T4 concentration is not affected by the protein level in the blood, it is thought to be more reliable than total T4 in evaluating thyroid activity (Dodds and Laverdure 2011). Free T4 concentration is less likely to be affected by non-thyroidal diseases or drugs (Bruyette 2020). In cases of hypothyroidism, both total T4 and free T4 concentrations are reduced. Although endocrinologists prefer the equilibrium dialysis (ED) RIA (radioimmunoassay) method for free T4 measurement, it is suggested that new technologies (improved analog RIA and non-RIA chemiluminescence and other methods) are alternative and accurate methodologies. These new measurement techniques are greener, faster and less costly because they do not require a radioisotope, Scott-Moncrieff 2007, Daminet 2010).

6.1.3. Total T3: Like total T4, total T3 exists in the bloodstream in bound and unbound T3 forms. Measurement of serum total T3 alone is not considered an accurate method for diagnosing canine thyroid disorders. This hormone reflects thyroid activity of tissue and is often simultaneously affected by non-thyroidal diseases. However, it is useful as part of a thyroid profile or health monitoring panel. For example, if total T4, free T4, and total T3 levels are all low, the dog is more likely to suffer from a non-thyroidal disorder rather than hypothyroidism. If a dog that does not receive thyroid supplementation has a high or very high total T3 level, it is very likely that the patient has circulating T3 autoantibodies. This falsely results in elevated T3 or free T3 levels (Scott- Moncrieff 2007, Dodds and Laverdure 2011).

6.1.4. FreeT3: Like free T4, it is less than 0.1% of freely circulating T3 in the blood and is biologically active. The body's level of free T3 alerts the pituitary gland whether it will produce more TSH. In euthyroid (normal thyroid function) dogs with increased metabolic demand, both to-

tal and free T3 levels may be slightly elevated. However, their surprisingly high or very high detection is due to T3 autoantibodies. In cases with hypothyroidism, both total T3 and free T3 levels are normal unless the dog has any other disease at the same time (Dodds and Laverdure 2011).

6.1.5. Thyrotropin (TSH) Stimulation Test: In the past, the TSH (thyrotropin) stimulation test was considered the gold standard for the evaluation of thyroid functions and the diagnosis of hypothyroidism. However, recently, thyroidal radioactive pertechnetate (TcO-4) measurement has become more valuable in differentiating primary hypothyroidism and nonthyroidal diseases (Diaz Espineira et al. 2007). This test is usually administered with recombinant human thyrotropin (rhTSH). The TSH stimulation test is usually done when T4 and TSH measurements give uncertain and questionable results. Although this test is useful in the evaluation of thyroid function, it is an expensive application. In the past, bovine TSH was used in the TSH stimulation test because bovine TSH is less affected than total T4 in euthyroid disease. However, this test is not used because it is expensive and bovine TSH is difficult to obtain (Boretti et al. 2006, Daminet 2007, Mooney 2011, Mooney and Shiel 2012)

In the measurement with bovine TSH, the protocol is firstly measuring the serum total T4 concentration and then intravenous TSH of 0.1 IU/kg (maximum dose is 5 units). It is waited for 6 hours to determine the serum total T4 concentration for the second time (Scott-Moncrieff 2007).

For dogs suspected of hypothyroidism, recombinant human TSH (rhTSH) is convenient and helpful. In the implementation of this test, the protocol again relies primarily on the measurement of serum T4 concentration. Serum total T4 concentration is measured before and 6 hours after administration of human TSH 50-100 μg (micrograms or mcg) intravenously. If the serum T4 concentration before and 6 hours after TSH administration is lower than the reference values, a diagnosis of hypothyroidism is made. If the serum total T4 concentration is above 2.5-3 nanogram(ng)/dL in the sample 6 hours after TSH administration, the diagnosis of euthyroid syndrome is made (Scott-Moncrieff 2007, Daminet 2010).

6.2. Histopathology

A biopsy from the thyroid gland is usually taken for definitive diagnostic procedure to determine the pathology of the gland. However, biopsy does not provide enough information about the functional capacity of the thyroid gland. The thyroid gland consists of surrounded follicles by a membrane. The wall of the thyroid follicle consists of a single layer of epithelial cells that are cuboid (cuboidal) when non-active and cylindrical (columnar) when active (Feldman and Nelson 2004). The lumen of the thyroid follicle contains viscous and colloid gel. This gel contains secret-

ed thyroglobulin by follicular cells of thyroid. However, it may be difficult to distinguish from normal histological changes to secondary hypothyroidism, follicular cell hyperplasia, primary atrophy and other diseases. Histopathology is important in differentiating central hypothyroidism and congenital hypothyroidism due to TSH/TRH deficiency with primary thyroid dysgenesis. Histopathology of the pituitary is helpful in evaluating the thyrotropic (thyrotropic) reserve of the adenohypophysis and hypothalamus. However, this is not possible ante-mortem. Case reports of both dogs and cats with congenital hypothyroidism with goiter show diffuse hyperplastic parenchyma with small amounts of colloid deposits, with a small follicular acini line with cuboidal epithelial cells as histopathological findings (Jones et al 1992, Pettigrew et al 2007). The characteristic evidence of TSH stimulation is irregularly shaped nuclei. The colloid is pale and slightly eosinophilic. Normal-appearing follicles with large columnar epithelial cells and many eosinophilic colloids may be few. The rate of normal follicles is most likely related to the degree of blockade in thyroid hormone synthesis. Primary congenital hypothyroidism shows variable histopathological findings (Bajonic et al. 2011). For example, hypoplasia of the thyroid gland is characterized by loss of glandular tissue and its replacement by adipose tissue. suggesting an inactive thyroid gland, and by little eosinophilic colloid. Unfortunately, these investigators did not identify the histological findings in the pituitary. In congenital hypothyroidism in Scottish Deer-hound puppies, the follicles are small, cuboidal cells and colloid-free. However, active thyrotropic cells in the adenohypophysis are characterized by an increase in the number of basophilic cells with large vacuoles containing few cytoplasmic granules. This finding suggests that thyrotrophs secrete large amounts of TSH. However, the reason for the inactivity in the thyroid gland may be due to the defect in TSH molecules or TSH receptors (Bajonic et al. 2011).

7. Sub-clinical Hypothyroidism

In some dogs with depressed thyroid function, laboratory results may still be within normal reference values. These dogs suffer from subclinical hypothyroidism. Subclinical hypothyroidism is a controversial topic in veterinary medicine. Because of dogs with borderline-low hypothyroidism often show none of the signs of hypothyroidism (skin and fur problems, weight gain and lethargy). However, exaggerated behavioral changes such as aggression and the inflammatory bowel disease in these animals suggest subclinical hypothyroidism.

It is important to understand how to diagnose subclinical hypothyroidism. It is divided by the upper and lower limits of the laboratory's normal reference values, and the value obtained represents the value in

normal healthy dogs. If the measured sample of the dog with suspecting hypothyroidism is in the normal reference values but below this value, it is considered subclinical hypothyroidism. This summary can be calculated as follows;

7.1. Foods That Inhibit Thyroid Hormone Production

Some foods naturally contain ingredients that can disrupt hormone production from the thyroid gland. These ingredients are called goitrogens and take this name from goiter, which means enlargement of the thyroid gland. The two main groups of foods containing goitrogens are soy products and cruciferous vegetables. Also, among the cereals, millet is slightly goatrogenic. If a dog is fed these foods, albeit limited, the dog will show clinical findings of thyroid imbalance. Studies have shown that the substance in soy that impairs thyroid function is isoflavones. Soy isoflavones can inhibit thyroid peroxidase (TPO) enzyme activity. This enzyme is found in the thyroid follicles and is responsible for the conversion of T4 hormone to T3 hormone. Soybean, which is used as a source of high quality vegetable protein in commercial pet foods, is interpreted as a potential cause of the increase in feline hyperthyroidism all over the world, raising the question whether this diet is a goitrogenic diet. Therefore, iodine deficiency increases the goitrogenic effect of soy, while the addition of iodine (appropriate amount of seaweed or sea kale) is protective. If hypothyroidism is suspected in a dog, vegetables such as broccoli, cauliflower, Brussels sprouts, cabbage, melon, kale (a vegetable that looks like cabbage), mustard, kohlrabi, turnips and radishes should be avoided. (Dodds and Leverdure 2011)

7.2. Factors Affecting Serum T3 and T4 Concentration

Various diseases and medications can affect thyroid function. Age, breed and many psychological factors can also affect thyroid function. Some dog breeds have lower thyroid hormone levels than normal. Total serum T4 level in Greyhound dogs is less than other breeds and approximately half of other breeds (Daminet 2006). Thyroid hormone concentration also changes with age. Total T4 hormone concentration begins to decrease from the age of 6 years. For this reason, tyrotropin (TSH) testing should be done less frequently in older ages and should be interpreted carefully. Except for Greyhound breed dogs, total T4 concentration is also low in Irish wolfhound, Scottish deerhound, Basenji, Golden retriever dogs. Therefore, scintigraphy and rhTSH stimulation test are recommended in these breeds (Daminet 2010).

8. Euthyroid syndrome

Although thyroid functions are normal, T4 and T3 levels are suppressed during diseases not related to the thyroid gland. Unbalanced diet and the use of certain drugs result in lower thyroid hormone production. Thus, the ability of plasma proteins to bind to T4 and T3 is decreased. The level of free T4 and free T3 concentrations increases. As a result of increasing free T4 and T3 concentration and negative feedback, TRH and TSH release are decreased. As a result, serum T4 and T3 levels decrease, but free T4 and free T3 levels are normal (Aytuğ 2011).

Thyroid hormone concentration is often low in diseases not related to the thyroid gland (diabetes mellitus, liver diseases, hyperadrenocorticism, renal and heart failures). This cases are called euthyroid sick syndrome. This change reflects the physiological adaptation of the organism due to the decrease in the energy requirement in the tissues. Thyroid hormone administration and treatment are not recommended for such patients (Daminet 2010).

8.1. Glucocorticoids: (prednisone, prednisolone): Glucocorticoids cause a direct decrease in TRH and TSH secretion by the mechanism mentioned above in total T4/T3 levels.

The results obtained in the tests performed 1 month after the discontinuation of the drug in patients receiving glucocorticoids may reflect the true values.

8.2. Other drugs

Sulfonamides: Using sulfonamides over 4-6 weeks results in decrease in thyroid hormone levels.

Phenobarbutals: Slight decrease in TT4 and FT4 levels in long-term treatment; TSH is normal in most dogs but increased in a small number of dogs.

Carprofen: Slight decrease in TT4, normal in FT4.

Clomipramine: Slight decrease in TT4 and FT4, TSH remains at normal levels.

9. Causes of Misunderstanding of Thyroid Diseases in Dogs

In the past, human test protocols were used in the diagnosis of hyper- or hypothyroidism in dogs. T4 level was measured in these test protocols. However, it has been determined that T4 measure alone is insufficient in the diagnosis of hypothyroidism in dogs and it may cause misdirections. Because the half-life of T3 and T4 is faster in dogs. Compared to humans, the half-life of T4 in dogs is 12-16 hours, while in humans it is 5-7 days.

The half-life of T3 is 6-8 hours in dogs and 24 hours in humans. This means that the active thyroid hormone concentration in the bloodstream of dogs is constantly low compared to humans. This can result in dogs being misdiagnosed with hypothyroidism frequently. In addition, the reference of normal thyroid values of healthy adult animals are similar for most dog breeds but may vary depending on breed, age and lifestyle. For example, puppies have higher basal thyroid levels than adults. Optimal thyroid levels in puppies are 50% higher than in adults. On the other hand, the basal metabolism of older animals is often low. Therefore, optimal thyroid levels of older dogs are usually close to or slightly lower than the reference values. Similarly, large breeds of dogs have lower basal thyroid levels naturally. Sighthounds are the dog breeds with the lowest thyroid levels. Dodds and Laverdure 2011).

10. Hypothyroidism Treatment

There are few options for the treatment and monitoring of dogs with hypothyroidism. Therefore, thyroxine (thyroxine; T4) treatment should be performed if the diagnosis of hypothyroidism is correct and definitive. When starting thyroxine treatment in dogs, many factors such as age, weight, breed and lifestyle should be considered. Synthetic thyroid hormones are more recomended because it long action. Sodium levothyroxine as synthetic T4 is the first treatment option. The half-life of levotroxine is 12-16 hours and peak concentration is reached within 4-12 hours after administration. The therapeutic dose in dogs is much higher than in humans because oral absorption in dogs is low. Levotroxine is absorbed from the gastrointestinal system through the ilium and colon. Determining the appropriate hormone replacement therapy in dogs is vital. To determine this dose, blood tests are done regularly every 6-8 weeks. Once the appropriate dose has been determined, these tests are repeated at least once a year. If the dose of the drug is determined by a specialist, it is extremely reliable. Otherwise, excessive thyroxine intake will cause thyrotoxicosis (thyroxine overdose). In case of overdose, do not panic. Because the half-life of thyroxine is short (12-16 hours). At the end of this period, the metabolism returns to normal. Increased activity, rapid breathing and excessive thirst are determined as signs of thyrotoxicosis in patients. The recommended initial dose of treatment in hypothyroidism is 20 mcg/kg twice a day, ie 0.02 mg/kg. In other words, the dose of thyroxine is 0.1 mg per 4.5-6.5 kg body weight. This dose is administered orally twice a day. A dog weighing 30 kg should be given 0.5mg of thyroxine every 12 hours. The patient should be reassessed on the basis of serum TT4 concentration and clinical findings within 1-2 months after initiation of therapy. In patients with heart problems, cardiac irregularity (destabilization) may

occur if an increase in metabolic rate occurs after administration of the initial dose. In such patients, half the starting dose is recommended. After drug administration, the blood hormone level reaches its peak concentration after 3-4 hours, and at the end of this period, the interpretation of the total T4 concentration is required. If the total T4 level is below normal, the dose of the drug should be increased, if it is above normal, the dose should be reduced. Controls should be made until the T4 level becomes stable (Dixon et al. 2002, Scott-Moncrieff 2007, Tilley and Smith 2008, Le Traon et al. 2009, Aytuğ 2011, Dodds and Leverdure 2011, Scott-Moncrieff 2012).

Synthetic triiodothyronine (T3) can be used as an alternative to treatment. This drug is used in cases that do not respond to two different levotroxines and cannot reach adequate T4 levels. Failure to respond to levothyroxine may result from impaired T4 absorption in the gastrointestinal tract. Synthetic T3 is not recommended as initial therapy. It is not used because serum T4 level is low and T3 level is normal. Since serum T4 concentration is important in the feedback regulation in the hypothalamic-pituitary-thyroid axis, it may cause iatrogenic thyrotoxicosis. The initial dose of this drug is 4-6 mg/kg orally divided into 3 doses per day. The half-life of T3 is 5-6 hours. In addition, the combined use of T3 and T4 preparations is not recommended. General condition and mental activity level improve after 1-2 weeks at the beginning of the treatment. Dermatological and neurological findings improve slowly and within 4-6 weeks. It takes 2-3 months for the hairs to regrow because the first hairs can be atypical. Reproductive disorders get better even later. Serum T4 and T3 concentrations inform the clinician about the treatment, dose and frequency of use of the drug. For measurement of serum T4 concentration, a blood sample should be taken 4-6 hours after the last drug intake. Both T4 measurement should be within normal ranges and T4 level should be slightly higher than normal after drug use (Dixon et al. 2002, Scott-Moncrieff 2007, Tilley and Smith 2008, Le Traon et al. 2009, Aytuğ 2011).

In addition, the K value has recently been calculated for the diagnosis of hypothyroidism and differentiation from euthyroid dogs.

K = [9.0 x free T4 (ng/dl)] – [0.026 x cholesterol (mg/dl)]

A K value less than -4 usually indicates hypothyroidism. In animals with a K value between -4 and +1, the TSH stimulation test should be performed. In dogs and cats with skin problems, weight gain, epilepsy, von Willebrant disease, low T3 and T4, K value <-4 and negative TSH stimulation test indicate hypothyroidism, while K value >+1 (>5 years old) , >3 (1-5 years old) and a positive TSH stimulation test are considered normal. In patients with a K value between -4 and +1, adequate response

to the TSH stimulation test indicates hypothyroidism, whereas if there is no adequate response to the TSH test, it is said to have hypothyroidism. (Larsson 1988, Ferguson 1994).

11. CONCLISION

Treatment of hypothyroidism in dogs requires lifelong therapy with oral thyroid hormone replacement. It should be noted that drug tolerance may change over time. For this reason, it may be necessary to adjust the dose of the drug used by your dog from during the treatment. During this time, it is recommended to measure of blood thyroid levels checked every 6-12 months. Not giving less or too much thyroid hormone during treatment is another important thing to be aware of. Once your dog's thyroid levels return to normal, your dog may lose weight, his body condition score may normalize, and he will likely have more energy. Although it may take months for your dog's hair to regrow, he will likely experience an improvement in his skin and coat over time. Thus, the quality of life and comfort will also increase.

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Chapter 13

AN EVALUATION OF RECEPTIVE AND EXPRESSIVE LANGUAGE SKILLS IN CHILDREN WITH HEARING LOSS

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INTRODUCTION

The main purpose of education for children with hearing loss is to help them develop their language skills and verbal communication skills as they get older. Sense of hearing is necessary for the development of children's receptive language and expressive language skills (Gunduz & Karabulut, 2015). Congenital or pre-linguistic hearing loss can affect a child's language development and cause the child to differ from his/her peers with normal hearing in cognitive, social, and emotional development (Dogan, Tufekcioglu & Nurhan, 2013). Even if hearing loss is mild, this situation causes articulation disorders, auditory memory difficulties, and delays in receptive language and expressive language development in children. As hearing loss increases, children's vocabulary and speech production decrease. Besides, children's speech perception, literacy skills, and academic success decrease with hearing loss (Marschark Rhoten, & Fabich 2007). This situation negatively affects children's literacy skills (Pistav Akmese & Acarlar, 2016). Hearing aids are widely used depending on the level of hearing loss and the location of the pathology (Sennaroglu, 2003). Early diagnosis of hearing loss, proper implantation, and starting auditory-verbal education in the early period are important in language development. Receptive language and expressive language skills of children with hearing loss increase in parallel with the use of aids (Turan et al., 2012). In addition to the use of hearing, intensive auditory-verbal education is an important factor in the verbal language acquisition of children with hearing loss (Kirk et al., 2000). Especially congenital severe and profound sensorineural hearing loss negatively affects a child's language development. Children's language deprivation causes serious delays in social, cognitive, and emotional development compared to their peers with normal hearing (Pistav Akmese, 2015).

It is stated that language skills and speech perception skills of children with hearing loss, who are in the risk group in terms of language development, increase thanks to the diagnosis with newborn hearing screening programs, hearing aids and cochlear implantation practices (Geers, 2003). Besides the developments in hearing aids and cochlear implantation technology, current studies state that difficulties in language skills in the early period negatively affect reading performances in later academic life (Kargin, Altun & Guldenoglu, 2021; Pistav Akmese, 2015).

Children with severe and profound hearing loss experience serious language problems in the early stages of their development. This situation constitutes a higher risk for children in terms of literacy difficulties in the following periods of life (Atlar & Uzuner, 2018). Even though children with hearing loss go through the same developmental stages as their peers with normal hearing (Uzuner et al., 2005), delays can be seen in the devel-

opment of communication, verbal language acquisition, problem-solving, and academic skills at different levels due to hearing loss (Truax, Foo & Whitesell, 2004; Luckner & McNeill, 1994). It has been reported that increasing early diagnosis, hearing aid use in the early period and early cochlear implant practices in recent years help support and develop verbal language skills of children with hearing loss (Uludag & Durmus, 2020). Nicholas and Geers (2007) and Pistav Akmese and Acarlar (2016) emphasized that children with severe hearing loss have a lower vocabulary than expected compared to their peers with normal hearing. Kyle and Harris (2010) stated that there is a strong relationship between reading skills and language skills of children and adolescents with severe hearing loss. At this point, the evaluation of language skills in children with hearing loss becomes prominent. This study aims to evaluate the language skills of children with cochlear implants and hearing aids and compare them with their peers with normal hearing (NH). The study sought answers to the following sub-questions regarding this general purpose:

Q-1) Do the language test results of children with hearing loss (CI+HA) and NH differ?

Q-2) Do the language test results of children with CI, HA, and NH differ?

Q-3) Do the language test results of children with CI and HA differ?

Q-4) Do the the language test results of the children who had CI operation before and after the age of 2 differ?

METHOD

This section of the study that aims to investigate the language skills of children with hearing loss and normal hearing includes the research model, study groups, test materials, data collection, and data analysis.

Research Model

The comparative descriptive research method was used in the comparative examination of language skills of children with hearing loss and normal hearing.

Study Groups

The study group included 50 children with hearing loss [35 with cochlear implants (CI) and 15 with hearing aids (HA)] and 50 children with normal hearing (NH), 100 children in total, who are between the ages of 4;2-7;10 and who continue inclusive education in private special education and rehabilitation institutions and preschools and primary schools affiliated to the Ministry of National Education in Izmir province. The

children with hearing loss and normal hearing were matched in accordance with the chronological age (± 3 months) and gender (Boons et al., 2013). The analysis conducted showed that there was no significant difference between the groups in terms of age and gender (t=0.000, p>.05). The inclusion criteria for the children with hearing loss were having been using CI or HA for at least 1 year, receiving auditory-verbal education, not having a disability other than hearing loss according to the information received from the family, having Turkish as their native language, and volunteering to participate in the study. Table 1 shows the distribution of children who participated in the study by age and gender.

Table 1: *Distribution of Children who participated in the Study by Age and Gender*

		CI	HA	NH
Gender				
	Female	19	10	29
	Male	16	5	21
		M±SD	M±SD	M±SD
Age (Years)		5.91±1.15	6.03±1.01	5.92±1.10

The study consisted of 19 female and 16 male children in the CI group, 10 female and 5 male children in the HA group, and 29 female and 21 male children in the NH group. The mean chronological age of the children were 5.91±1.15 (min:4;02-max:7;10) in the CI group, 6.03±1.01 (min:4;04-max:7;10) in the HA group and 5.92±1.10 (min:4;02-max:7;10) in the NH group.

Demographic characteristics of the children with hearing loss who participated in the study were given in Table 2.

Table 2: *Demographic Characteristics of Children with Hearing Loss*

Variables		CI		HA	
		N	%	N	%
Hearing loss diagnosis age (months)	Newborn Screening	5	14.3	-	-
	3-6	7	17.1	3	20
	6-12	11	34.3	1	6.7
	13-24	8	22.9	6	40
	25-36	4	11.4	5	33.3
Ear with CI					
	Right ear	28	93.3		
	Left ear	2	6.7		

As seen in Table 2, of children with CI, 14.3% were diagnosed with newborn hearing screening, 17.1% were diagnosed between 3-6 months, 34.3% were diagnosed between 6-12 months, 22.9% were diagnosed between the ages of 1-2, and 11.4% were diagnosed between 25-36 months. Of children with HA, 20% were diagnosed between 3-6 months, 6.7% were diagnosed between 6-12 months, 40% were diagnosed between the ages of 1-2, and 33.3% were diagnosed between 25-36 months. 93.3% of children with CI use CI on their right ears and 6.7% use CI on their left ears. When looking at the brands of CI used by the children who had CI operation, 45.7% use Medel, 31.4% use Nucleus, and 22.9% use Advance Bionics.

Children who had CI operation had used hearing aids between 4 month to 4 years (Mean=1.60) before the operation. Children's age of operation varies between 1;00 and 5;10 years (Mean=2.43). The duration of CI use varies between 1 year and 7 years (Mean=3.91), the age of starting auditory-verbal education is between 6 months and 3 years (Mean=1.52) for the CI group and between 1 year and 4 years (Mean=2.53) for the HA group. Children's duration of HA use varies between 1 year and 6 years (Mean=4.05). All children with CI and HA started receiving auditory-verbal education just after they started using aids and all children have been receiving auditory-verbal education.

Table 3 shows the distribution of parents of the children included in the study group by education level.

Table 3: *Distribution of the Parents of Children in the Study Group by Education Level and Profession*

	CI		HA		NH							
	Mother M±SD		Father M±SD		Mother M±SD		Father M±SD		Mother M±SD		Father M±SD	
Age	34.71±5.47		36.80±5.23		35.13±5.79		40.20±6.77		37.66±3.59		40.68±4.87	
	N	%	N	%	N	%	N	%	N	%	N	%
Education Level												
Primary School	17	48.6	9	25.7	11	73.3	8	53.4	14	28	11	22
Secondary School	2	5.7	3	8.6	-	-	3	20	-	-	-	
High School	10	28.6	15	42.9	1	6.7	2	13.3	11	22	11	22
University Profession	6	20.0	8	22.9	3	20	2	13.3	25	50	28	56
Civil Servant	4	11.4	11	31.4	2	13.3	1	6.7	24	48	17	34
Employee	1	2.9	14	40	-	-	8	53.3	4	8	10	20
Self-employed	2	5.7	10	28.6	-	-	6	40	8	16	23	46
Housewife	28	80	-	-	13	86.7	-	-	14	28	-	-

When examining the mothers by their education levels, 48.6% of the mothers of children with CI, 73.3% of the mothers of children with HA, and 28% of the mothers of children with NH are primary school graduates. When examining the fathers by their education levels, 42.9% of the fathers of children with CI are high school graduates, 53.4% of the fathers of children with HA are primary school graduates, and 56% of the fathers of children with NH are university graduates. While the mothers of children in the CI and HA groups are mostly primary school graduates, the mothers of the children in the control group are mostly university graduates. When examining the mothers by their professions, 80% of the mothers of children with CI, 86.7% of mothers of children with HA, and 28% of children with NH are housewives. 40% of the fathers of children with CI and 53.3% of the fathers of children with HA are employees, and 46% of the fathers of the NH group are self-employed.

It was found that there was no significant difference between the mothers of children with hearing loss and mothers of children with NH ($X2(2)=3.34$, $p=.188$) in terms of mothers' education level, which is an important factor in the language development of children, and there was no significant difference between the fathers of two groups ($X2(2)=4.591$, $p=.101$).

In the study, after obtaining necessary permissions from the families, first, the demographic information form containing the information of the children and families was filled out. Hearing tests of the children with hearing loss were available as they had regular check-ups. Hearing tests were applied to the children in the NH group. The hearing test was conducted in a quiet room at the school where children with NH continéue their education, using a amplaid 171 S Type 3 IEC 645 and earphones. Children who were below 20 dB HL at all frequencies between 500-4000 Hz were included in the study. After the hearing test, language test was applied to the children.

Test materials

Turkish test of early language development: Turkish adaptation of "Test of Early Language Development-Third Edition (TELD-3) which was developed by Hresko, Redid, and Hammill (1999) to determine receptive and expressive language skills of the children was utilized in the study (Topbas & Guven, 2011). Turkish test of early language development (TEDIL) is a norm-based test that measures the verbal language development of children who are between the ages of 2;0-7;11 and whose native language is Turkish. In the test, the child is asked to show pictures, follow verbal instructions, and give verbal responses to questions. Receptive language and expressive language scores are obtained in the test. The

test results distinguish between children who show normal language development and children with language disorders (Topbas & Guven, 2013).

Data collection

The evaluations of the children were conducted in two sections by giving a break on the same day at the centers or schools where they studied. The evaluations were carried out individually by the researcher in a quiet room. The rules that should be followed were explained to the children before the evaluations. The instruction was repeated when the child could not understand it. TEDIL took approximately 30-45 minutes. While applying TEDIL to the young children, a 5-minute break was given between receptive language and expressive language tests.

Data Analysis

The research data were analyzed using SPSS 23.0 package program. Independent sample Kruskal Wallis test, Mann Whitney U test, and t test were used to analyze whether the children with hearing loss and NH differ the language development test TEDIL.

RESULTS

Results of the statistical analysis conducted in line with the purposes were included in this section of the study that aims to evaluate the language skills of children with cochlear implants (CI) and hearing aids (HA) and examine them in comparison with their peers with normal hearing (NH).

Do the receptive language and expressive language test results of children with CI+HA and NH differ?

Independent sample t test was used to examine whether there was a difference between the groups in terms of receptive language and expressive language scores obtained from the TEDIL language development test applied. The results are shown in Table 4.

Table 4: *Independent Sample t Test results*

TEDIL	Groups	N	M±SD	t	p
Receptive Language	Hearing Loss	50	86.220± 10.56	-10.83	.000*
	Normal Hearing	50	105.54± 6.90		
Expressive Language	Hearing Loss	50	84.66 ±11.47	-10.35	.000*
	Normal Hearing	50	105.24± 8.12		

*p<.05

As seen in Table 4, the receptive language and expressive language mean scores of the children with CI are lower than the children with nor-

mal hearing. According to the independent sample t test results, there was a significant difference between the groups in favor of children with normal hearing in receptive language [t=- -10.83, p=.000] and expressive language [t=-10.35.64, p=.000].

Do the receptive language and expressive language test results of children with CI, HA, and NH differ?

Kruskal Wallis test was used to analyze whether there was a difference between the groups with CI, HA, and NH in terms of receptive language and expressive language scores obtained from the TEDIL language development test applied. The test results are shown in Table 5.

Table 5: *Kruskal Wallis Test Results*

Kruskal Wallis Test Results

TEDIL		Receptive Language				Expressive Language			
	N	Mean Avarage	df	χ2	p	Mean Avarage	df	χ2	p
CI	35	27.14				29.49			
HA	15	32.70	2	6,386	.000	27.63	2	55,572	.000*
NH	50	72.19				72.07			

*p<.05

As seen in Table 5, when comparing groups with CI, HA, and NH, there was a significant difference between the groups in TEDIL receptive language (χ2=6.386, p=.000) and expressive language (χ2=55.572, p=.000) scores. Mann-Whitney U test was used to determine which groups cause this difference. A significant difference was found between groups when both groups with CI and HA were compared with the group with NH in pairs.

Do the receptive language and expressive language test results of children with CI, HA, and NH differ?

Mann-Whitney U Test was used to examine whether there was a difference between the groups with CI and HA in terms of receptive language and expressive language scores obtained from the TEDIL language development test applied. The results are shown in Table 6.

Table 6: *Mann-Whitney U Test Results*

TEDIL	CI M±SD	HA M±SD	U	p
Receptive Language	85.34 ±10.26	88.27± 11.33	222.50	.397
Expressive Language	84.74 ±12.24	84.47± 9.83	254.50	.865

*p<.05

As seen in Table 6, it was found that there was no significant difference between the groups with CI and HA in TEDIL receptive language (U=222.50, p=.397) and expressive language (U=254.50, p=.865) scores. This finding shows that the receptive language and expressive language skills of the children with CI and HA are similar.

The children in the CI, HA, and NH groups were compared with the norm values according to their place and levels in the general distribution measurement in accordance with the receptive language and expressive language scores obtained from the TEDIL language test applied. The results are shown in Table 7.

Table 7: *Place and Levels of Children with CI, HA, and NH in the Language Scores*

TEDIL		CI		HA		NH	
		N	%	N	%	N	%
Receptive Language	Above Average	-	-	-	-	13	26
	Average	14	40	8	53.3	37	74
	BelowAverage	9	25.7	4	26.7	-	-
	Weak	12	34.3	3	20	-	-
Expressive Language	Above Average	-	-	-	-	17	34
	Average	13	37.2	7	46.7	33	66
	Below Average	9	25.7	5	33.3	-	-
	Weak	10	26.6	3	20	-	-
	Very weak	3	8.6	-	-	-	-

When examining Table 7, it is seen that the TEDIL receptive language and expressive language scores of the entire NH group were on average and above average. In the group with CI, 40% showed age-appropriate performances in receptive language and 37% showed age-appropriate performances in expressive language. Approximately 60% of the children showed performances below average, weak, or very weak performances. It was seen that 53.3% of the children in the HA group showed average performance in receptive language and 46.7% showed average performances in expressive language; others showed performances below average or weak performances. In this study, it was seen that the HA group showed better performances in receptive and expressive language while there was no statistically significant difference between the group with CI and HA.

Do the receptive language and expressive language test results of the children who had CI operation before and after the age of 2 differ?

Mann-Whitney U Test was used to examine whether there was a difference between the receptive language and expressive language scores of children who had CI operation before and after the age of 2. The results are shown in Table 8.

Table 8: *Mann-Whitney U Test Results*

TEDIL	Groups	N	Mean Avarage	Rank Total	U	p
Receptive Language	Below the age of 2	16	21.19	339.00	101.00	.095
	Above the age of 2	19	15.32	291.00		
Expressive Language	Below the age of 2	16	24.63	394.00	46.00	.000*
	Above the age of 2	19	12.42	236.00		

*p<.05

In Table 8, when comparing the scores received by the children in TEDIL language measurements in accordance with the age of CI operation, it was found that the age of operation does not pose a significant difference in receptive language scores but it causes a difference in expressive language scores (U=46.00, p=.000). This finding shows that having the operation before the age of 2 is effective in increasing the expressive language skills of children with CI.

DISCUSSION

As a result of the study that aimed to evaluate the language skills of children with cochlear implants (CI) and hearing aids (HA) and to compare them with their peers with normal hearing (NH), it was found that the receptive language and expressive language mean scores of the children in the CI group are lower than children with normal hearing. In this study, it was found that 40% of children with CI show age-appropriate language characteristics while 60% are below the age-appropriate level; 50% of children with HA show age-appropriate performances while the other half were below the age level. Different results have been obtained in the studies that compare children with hearing loss with children with normal hearing. Geers et al. (2009), in a study that examined 153 children with CI, stated that 50% in receptive language vocabulary, 58% in expressive language vocabulary, 47% in receptive language, and 39% in the expressive language received age-appropriate scores. Schorr et al. (2008) found that 36% of the CI group and 92% of the NH group showed age-appropriate performances in all tests, supporting the results of this study. Pistav Akmese and Acarlar (2016) found that 63% of children with CI in receptive language and 50% in expressive language showed age-appropriate performances. In another study that shows that there are different language profiles in children with CI, Duchesne et al. (2009) compared the vocabulary, morpheme, and syntactic structures of 27 children with CI between the ages of 3-8. As a result of the study, it was stated that they showed different language profiles such as normal language development, language delay in all areas, normal vocabulary/semantic knowledge with delay in receptive language, and inconsistency between language areas. Reuterskiöld, Ibertsson, & Sahlén (2010), in a study they investigated the narration skills of children with hearing loss, evaluated the narration skills of 18 children who use hearing aids due to mild-moderate bilateral sensorineural hearing loss using "One Frog Too Many" test. They stated that narratives of the children with HA included less content knowledge and they tend to use less developed conjunctions than their peers with NH. Le Normand et al. (2003), in their longitudinal study, evaluated 17 children with CI in their second and third years of CI use. They found that some children reached a normal or near-normal language level, some children

had a vocabulary learning disability, and some children produced a few words or could not produce any words and showed serious delays. In conclusion, in these studies, it is seen that the language skills of the children with hearing loss differ and nearly half of them are behind their peers with normal hearing. These findings show that using only general language tests does not provide comprehensive information about which areas of language children have difficulty in and it is important to carry out detailed evaluations about components of language such as vocabulary, morpheme knowledge, and syntax and narrative skills of children. Besides, many factors such as the age of hearing loss diagnosis and hearing aid implementation, auditory-verbal education they receive, and inclusive education may affect the language skills of children.

Age of operation is the main factor that affects language skills. In this study, it was found that the age of operation does not pose a significant difference in receptive language scores but it causes a difference in expressive language scores. This finding shows that having the operation before the age of 2 is effective in increasing the expressive language skills of children with CI. Similarly, Pistav Akmese and Acarlar (2016) found that there was a significant difference in the expressive language scores of the NH group and CI groups who had the operation before and after the age of 2 and there was no significant difference in receptive language. They also found that there was a significant difference in TNW mean scores only in the A3 story between the two groups in terms of age of operation and children who had the operation before the age of 2 told complex stories with more characters and events. Similarly, it has been emphasized in the literature that child's age of operation is a significant factor that affects the post-operation success, the earlier the operation of the child, the faster the post-operation development observed, and children who were implanted with CI before the age of 2 reached the language level appropriate for their ages (Hammes et al., 2002; Hayes et al., 2009; Kirk et al., 2002; Miyamoto et al., 2003). Niparko et al. (2010) stated that children who had CI operation showed great development in expressive language and language comprehension and the best predictor of the postoperative language scores of the children is preoperative language skills. Svirsky et al. (2004) stated that as the age of CI operation increases, the expressive language scores decrease. Akin et al. (2009) expressed that factors such as preoperative aid use, preoperative language skills, and regular education affect the children's success. Pistav Akmese and Acarlar (2016) drew attention that early operation is only one of the factors that affect CI success and various factors such as the education received by the children, characteristics of the family, and inclusive education affect the success of the implant. In conclusion, evaluating the language development of children with hearing

loss in detail and developing their language skills will enable children to establish a more successful future both socially and academically.

Conclusion

The results of this study have shown the importance of determining and evaluating the purposes regarding receptive and expressive language skills in early auditory-verbal education programs. Because it is highly significant to evaluate early language skills which are a prerequisite for the academic skills of children with hearing loss and to support them with intensive auditory-verbal education starting from the early childhood period.

In line with the research findings, in future studies, the same study can be planned in longitudinal design and the language development of children with hearing loss can be followed. Thus, the effects of the other factors regarding the language development of children with hearing loss can be revealed. The relationship between auditory perception, speech perception, narration, early literacy, and mathematical reasoning skills as well as the receptive language and expressive language skills of hearing loss can be examined. The effect of an education program implemented to support language skills can be evaluated.

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Chapter 14

THE EFFECTS OF MINERALS AND HEAVY METALS IN DIFFERENT KINDS OF TABLE SALTS ON HEALTH

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Introduction

Salt is a natural additive stabilizing manifold physiological functions of living creatures and is essential to safeguard the continuation of life (Chander et al., 2020; Wulandari, 2017). Salt has been commonly utilized as a valuable spice and preservative from past to present (Aquilano, Otálora, Pastero, & García-Ruiz, 2016). Salt, which comprises of sodium chloride exists naturally in plenty of foods, including milk, eggs, and shellfish (Fayet-Moore et al., 2020). Salt is obtained from the boiling of sea and lake water or from various natural sources (mines) (Kuhn et al., 2020; McKillop & Aoyama, 2018). Discrepancies may be encountered in mineral values of salts procured in different ways, and potential particularities in heavy metal ratios of salts can be detected because of pollution.

Minerals found in salts bear diverse consequences for health. Besides minerals necessary for the body, salts contain heavy metals. Heavy metals are defined as metals with high concentration, and they produce a toxic effect even when exposed to low concentration (B. Duwiejuah, J. Cobbina, & Bakobie, 2017). Some metals such as cadmium, lead, aluminum, mercury, and manganese, despite not having biological functions remain toxic when exposed to. By acting as cofactors of enzymes, metals like iron and copper get physiologically involved in the biological processes of living organisms (Woimant & Trocello, 2014).

Heavy metals play a crucial role in the physiology and pathology of the nervous system (B. Duwiejuah et al., 2017). They can adversely affect the neurodevelopmental process (R. C. Brown, Lockwood, & Sonawane, 2005). Various studies demonstrate that there is a connection between heavy metals and neurodegenerative disorders such as Alzheimer's and Parkinson's disease (R. C. Brown et al., 2005; Hussien et al., 2018). Aluminum increases the risk of Alzheimer's by amplifying the expression of genes such as acute phase protein (APP), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) (Cao, Wang, Xiu, Zhang, & Li, 2017; Lukiw, Percy, & Kruck, 2005). Cadmium and fluorine impinge upon the hippocampus, which occupies a place in the medial temporal lobe of the brain and is instrumental in memory and navigation. In addition, free radical formation of cadmium and fluorine in the body leads to an inflation of lipid peroxidation (R. C. Brown et al., 2005; B. Wang & Du, 2013). In a study by Rossignol et al., it is acknowledged that such heavy metals may cause autism spectrum disorder (ASD), the prevalence of which is escalating worldwide. Furthermore, it is asserted that the concentration of heavy metals in blood, urine, hair, brain, and tooth samples of children with ASD is higher than it is in normal individuals (Rossignol, Genuis, & Frye, 2014).

The reason for the growing interest in different types of salt today is its variety of uses in the food industry and the belief that some salts are healthier (Carapeto, Brum, & Rocha, 2018a). Salt is found naturally in seas, brine lakes and groundwater sources as mineral halides. Differences in chemical compositions of salts result in diversity of taste. The mineral content of salts differs depending on the source, and it is possible to supplant certain salts with others to promote a reduction in the sodium content of a diet (Carapeto, Brum, & Rocha, 2018b). Industrialization has reinforced the interest in several types of salts, besides inducing the spread of heavy metal pollution in air, water and soil (Vats, Bhargava, Road, & Gupta, 2017) Resulting situation pollutes drinking water, impairs air quality and disturbs public health with the impact it has on foods consumed by living organisms. Environmental changes in nature engender phenotypic and genotypic alterations that affect the health of individuals (Muhammad, Shah, & Khan, 2011; Tchounwou, Yedjou, Patlolla, & Sutton, 2012). This study was conducted to examine 6 different salts, namely lake salt, pink Himalayan salt, white Himalayan salt, sea salt, iodized salt, and local rock salt, and to analyze their mineral and heavy metal contents.

Material and Methods

Study design: 6 different salt samples, namely lake salt, pink Himalayan salt, white Himalayan salt, sea salt, iodized salt, and local rock salt, were obtained from local markets in Turkey.

Measurements: The samples were stored at room temperature until their analysis (Figure 1). The samples were examined for their V, Co, Cu, Zn, Mg, K, Na, Ni, As, Cd, Pb, Fe, Al and Hg contents. Firstly, the salt samples were weighed. Then, they were diluted with 20 ml of 2% HNO3 and dissolved in a hot water bath for 1.5 hours (50ºC and 100 rpm) [(0.2g) diluted with 20 ml of 2% nitric acid (HNO3).] Mineral determinations were made using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and Atomic Absorption Spectroscopy (ASS) instruments. The results were expressed in terms of (ppm).

Data analysis: Statistical analysis of the data was performed using SPSS 20 (SPSS,Inc., Chicago, Ill., USA).

Results

When the results were reviewed, the salt with the highest nickel (Ni) heavy metal mineral value was the lake salt, and the one with the least nickel was the white Himalayan salt. A study of the aluminum (Al) values revealed the iodized salt to be having the highest amount. It was also observed that the sea and pink Himalayan salts contain similar amounts of aluminum. Aluminum could not be detected in the rock salt and the white

Himalayan salt. The pink Himalayan salt was discovered as the salt with the highest iron (Fe) mineral. Iron was not encountered in the iodized salt and the rock salt. The highest arsenic (As) value belonged to the lake salt and the lowest to the iodized salt. In terms of cadmium (Cd) mineral value, it was seen that, apart from the iodized salt, all the salts were similar. The salt with the highest Cd value was the pink Himalayan salt and Cd could not be spotted in the iodized salt sample.

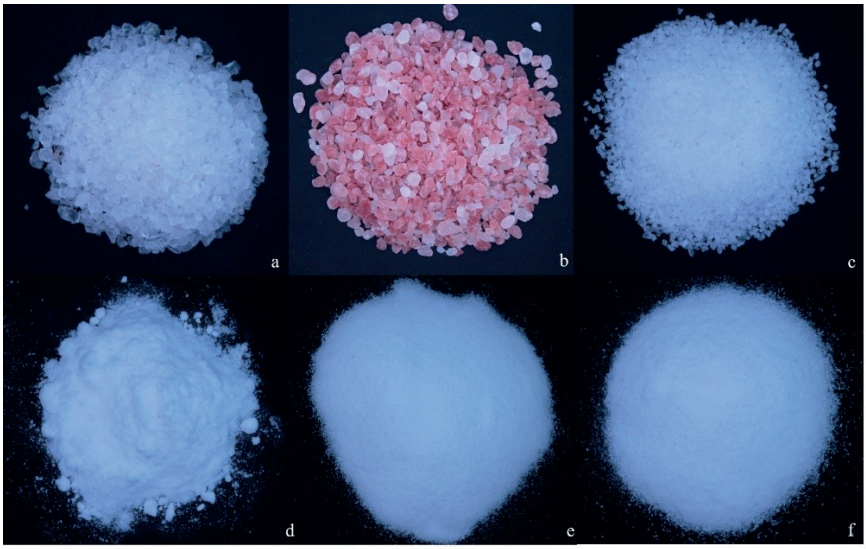

Figure 1. *Six different types of salt*
a:himalayan salt, b:pink himalayan salt, c:sea salt, d:rock salt, d:lake salt, e:ionized salt

The salt with the highest lead (Pb) was the white Himalayan salt, which was followed by the pink Himalayan salt, and the sea salt. The rock salt from Kırıkkale's Keskin district was found to be having the lowest lead value. Mercury (Hg) was not detected in any of the salts. Vanadium value was discovered to be similar in all of the salts examined. The kind of salt with the highest cobalt content was the pink Himalayan salt. The cobalt amounts of the other salts appeared to be similar.

The World Health Organization (WHO) recommends an average daily salt intake of 5g. Table 2 illustrates the amounts of minerals and heavy metals that exist in different salts of 5g which is the quantity recommended to be consumed daily. RDA and EAR values for minerals and upper limits for heavy metals are also illustrated.

Table 1. *Mineral and heavy metal values of the salts.*

Minerals	TYPE					
	Lake Salt	Pink Himalayan Salt	Rock Salt	Sea Salt	Iodized Salt	Himalayan Salt
Nutrients						
Cu(ppm)	0	0,8407	0,5806	0,5759	0,6533	0,5342
Zn(ppm)	0,0555	0,2259	0,1796	0,1055	0,1697	0,0906
Mg(ppm)	0,56639	5240,1	119,127	228,472	2,74826	207,317
K(ppm)	11,88179	2519,802	96,98254	188,5266	0	177,7004
Fe(ppm)	22,28529	2349,752	0	75,51767	0	76,80438
Na(ppm)	2621	2799,5	3033,5	2701	2891,5	2790
Heavy Metals						
Ni(ppm)	1,422	1,124	1,044	1,049	0,879	0,834
As(ppm)	1,387	1,122	1,043	1,052	0,919	1,001
Cd(ppm)	0,0503	0,0716	0,0538	0,0225	0	0,0401
Pb(ppm)	0,9821	4,63	0,4217	2,59	2,356	4,677
Al(ppm)	0,1088	0,3227	0	0,3671	2,2074	0
Hg(ppm)	0	0	0	0	0	0
V(ppm)	4,0175	4,3608	4,4175	3,9469	4,3554	4,6421
Co(ppm)	0,0041	0,0729	0,0116	0,0054	0,0031	0,0036

Table 2. *Mineral and heavy metal values of 5g salt.*

Minerals	TYPE						EAR (mg)	RDA (mg)	Upper Limit (mg)
	Lake Salt	Pink Himalayan Salt	Rock Salt	Sea Salt	Iodized Salt	Himalayan Salt			
	Mean Content in 5 g								
Nutrients									
Cu(ppm)	0	0,8407	0,5806	0,5759	0,6533	0,5342	1.2–1.7	-	10
Zn(ppm)	0,0555	0,2259	0,1796	0,1055	0,1697	0,0906	7,3	9.5 - 7.0	40
Mg(ppm)	0,56639	5240,1	119,127	228,472	2,74826	207,317	330	400	350
K(ppm)	11,88179	2519,802	96,98254	188,5266	0	177,7004	2800	3100-3500	4000
Fe(ppm)	22,28529	2349,752	0	75,51767	0	76,80438	8*18	8*10	45
Na(ppm)	2621	2799,5	3033,5	2701	2891,5	2790	460–920 (2000 [6])	-	-
Heavy Metals									
Ni(ppm)	1,422	1,124	1,044	1,049	0,879	0,834	-	-	1 [10]
As(ppm)	1,387	1,122	1,043	1,052	0,919	1,001	-	-	0.5[8]
Cd(ppm)	0,0503	0,0716	0,0538	0,0225	0	0,0401	-	-	0.5 [8]
Pb(ppm)	0,9821	4,63	0,4217	2,59	2,356	4,677	-	-	0.2 [8]
Al(ppm)	0,1088	0,3227	0	0,3671	2,2074	0	-	-	307
Hg(ppm)	0	0	0	0	0	0	-	-	0.01[8]
V(ppm)	4,0175	4,3608	4,4175	3,9469	4,3554	4,6421	-	-	1.8 [10]
Co(ppm)	0,0041	0,0729	0,0116	0,0054	0,0031	0,0036	-	-	-

EAR: *Estimated average requirement, RDA: Recommended daily allowance*

Discussion

It is widely acknowledged that minerals are indispensable for the physiological harmony of our bodies. Aluminum is among the minerals in the heavy metals group (Tayfur, Ünlüoğlu, & Bener, 2002). Taking high doses of aluminum; may cause tremor, convulsions, psychosis and other related neurological problems (Landry, 2014). It is also associated with neurological and bone abnormalities, Alzheimer's, Parkinson's diseas-

es and cognitive disorders (Greger, 1993; Greger, Sutherland, & Yokel, 1997; Krewski et al., 2007). In research conducted by Fayet-Moore et al., the amount of aluminum in different salt samples was found to be between 0 and 192.65 mg/kg (Fayet-Moore et al., 2020). In the present study, the salt which had the highest aluminum was the iodized salt (0-2.2 ppm). Although this amount is higher than the other salts, it does not exceed the daily average intake.

Nickel commonly finds its way into the biological system through food, water, skin contact, and air. In addition, its industrial use often leads to detrimental outcomes for organs such as kidney, lung, liver, and brain (Ijomone, Olatunji, Owolabi, Naicker, & Aschner, 2018; McDermott, Salzberg, Anderson, Shaw, & Lead, 2015). The presence of nickel in the body brings about an increase in the production of free radicals, begetting an upsurge of oxidative stress. Nickel's presence may also indirectly transform the DNA repair process by causing a change in protein structure (H. Chen et al., 2010). Multpile studies have examined the significance of Ni concentration in ASD disease. Al-farsi et al., in a study analyzed the amount of Ni in the hair samples of children with ASD, asserting that the amount of Ni in the hair samples of children with ASD was significantly higher than in normal children (H. Chen et al., 2010). In the study of Chen et al., it was affirmed that the prevalence of ASD is high in the children of parents living in areas with high atmospheric Ni (H. Chen et al., 2010). In the research by Fayet-Moore et al., the amount of nickel in Himalayan salts sold in Australia was found to be very low (Fayet-Moore et al., 2020). In this study, it was noticed that the amount of nickel in the salts was higher than the values found by Fayet-Moore et al.

Overexposure to heavy metals such as lead, arsenic, mercury, cadmium, chromium, may cause serious public health hazards, including cardiovascular diseases, developmental abnormalities, neurological and neurobehavioral disorders, diabetes, hearing loss, hematological and immunological disorders, and different types of cancer (Vats et al., 2017). Furthermore, with its bioaccumulation tendency, long biological half-life, ability to interfere with DNA repair mechanisms and formation of reactive oxygen species, cadmium is associated with various neurological diseases (Branca, Morucci, & Pacini, 2018; Iqbal, Zada, Mannan, & Ahmed, 2018). In the study of Soylak et al., the heavy metal contents of table salts found in Egypt and Greece were examined.(Soylak, Peker, & Turkoglu, 2008) In the examination, the Pb concentrations of the salts were noted to be between 0.54 and 1.64 µg/g. Additionally, Cheraghali et al., in the study they conducted on salts found in Iran, identified the amount of Pb as 0.438 µg/g (Nnorom, Osibanjo, & Ogugua, 2007). Similar to our study, Fayet-Moore et al. encountered the highest Pb value in pink Himalayan

salt (Fayet-Moore et al., 2020). Although this amount is higher compared to the other salts, special attention may be required even if it does not exceed the daily average intake. Moreover, Nnorom et al. reported that Cd levels in salts consumed in Nigeria were as high as 4.5 µg/g above the normal value (Nnorom et al., 2007). In this study, the salt with the highest cadmium mineral value was the pink Himalayan salt and unlike Nigeria, this value was within the normal range.

No disease has been detected in humans due to a deficiency of the trace element vanadium (Çevik, 2014). Fayet-Moore et al. found the vanadium value to be 0.12 mg/kg in their study (Fayet-Moore et al., 2020). In our study, the vanadium value was identified as similar in all the salts on average, and unlike Fayet-Moore et al., the value was between 4.0175 and 4.6421 ppm.

Cobalt is an essential element for all mammals, especially humans. Vitamin B12 contains approximately 4.5% cobalt. In diets, it is mainly found in the liver, bones and kidneys. Excessive iron intake can reduce the absorption of cobalt and induce its deficiency (Uyanık, 2000). The salt kind with the highest cobalt content was the pink Himalayan salt. The cobalt contents of the other salts appeared to be similar.

Copper is a crucial trace element in the body (Uyanık, 2000). Its deficiency can lead to various clinical conditions including prematurity, malnutrition, malabsorption, and chronic diarrhea (Aydın, Ulusoy, & Mocan, 1992). Also, exposure to toxicity from copper, lead, iron, and manganese has been shown to cause Parkinson's disease, sensory and motor disorders, cognitive dysfunction (e.g., dementia, epilepsy, amnesia), functional gait disorders, and postural balance problems (P. Chen et al., 2019; Iqbal et al., 2018; Tan, Cheng, Su, Chen, & Yang, 2021). Furthermore, epidemiological studies have demonstrated that for adults, chronic exposure to cadmium results in peripheral neuropathy, loss of concentration, and slowing of visual-motor function (Anyanwu, Ezejiofor, Igweze, & Orisakwe, 2018). In the study by Fayet-Moore et al., copper, chromium and manganese values in pink Himalayan salt were detected in negligible amounts (<1.00 mg/kg) (Fayet-Moore et al., 2020). During the analysis phase of this study, the copper mineral was not detected in the lake salt, and it was found in similar amounts in the other salt types. These amounts were in agreement with the study of Fayet-Moore et al. (<1ppm).

The presence of heavy metals such as mercury, lead, arsenic, nickel, copper and iron in body fluids such as urine, saliva, sweat, and fetal and infant tissues has been revealed (Cheraghali, Kobarfard, & Faeizy, 2010). Some toxic metals are transported throughout the body by ligands, while others diffuse as free ions through calcium and zinc membrane channels.

Due to the high permeability of the blood-brain-barrier and blood-cerebrospinal fluid interfaces in the prenatal period, heavy metal molecules are transported by the CNS and may cause neurotoxicity as a result (Azeh Engwa, Udoka Ferdinand, Nweke Nwalo, & N. Unachukwu, 2019; J. Wang et al., 2019).

Zinc, which is considered a relatively non-toxic metal, is essential for several biological reactions in the body and must be included in daily nutrition. Unlike metals such as copper (Wilson's disease) and iron (hemochromatosis), there are no known genetic abnormalities that cause excessive zinc accumulation in the body. The antioxidant effect of zinc, which is one of its most important tasks, makes it among the valuable minerals (Akdeniz, Kınık, & Yerlikaya, 2016; K. H. Brown, Wuehler, & Peerson, 2001; Hambidge, 2000; Walsh, Sandstead, Prasad, Newberne, & Fraker, 1994). In this study, zinc mineral was detected in all of the salts. It was observed that the pink Himalayan salt and the iodized salt have a higher amount of zinc compared to the other salts.

Iron, one of the most abundant metals in the human body, plays an important role in cellular processes such as DNA, RNA and protein synthesis. It is involved in electron transport, cellular respiration, cell proliferation and differentiation, and regulation of gene expression. In diets, especially liver, brain, and red meats contain high levels of iron (Lieu, Heiskala, Peterson, & Yang, 2001). High amount of iron taken orally acts on the gastrointestinal barrier with its caustic effect on the gastrointestinal tract mucosa and may lead to iron poisoning (Özgül et al., 2011). In addition, iron accumulates in the brain, especially in microglia and astrocytes, as well as in the form of ferritin in oligodendrocytes (Vats et al., 2017). It is stated that there is an increased iron concentration in the cortex and cerebellum in cases of preclinical Alzheimer's disease or mild cognitive impairment (Smith et al., 2010). Iron may have a direct effect on plaque formation through its impact on amyloid precursor protein, and it has been reported that iron accumulation in neurons may be the character of Parkinson's disease (Benarroch, 2009). In this study, the Himalayan salts were identified as the salts that contained higher amounts of iron mineral than the other salt types. In particular, the amount of iron in the pink Himalayan salt was noticed to be high, and no iron mineral could be detected in the rock salt and the iodized salt.

Sodium, along with other minerals such as chlorine and potassium, is called an electrolyte. It is crucial for electrical conduction in the body. Sodium deficiency is rare in living organisms (Ayaz, 2008). The World Health Organization stated the minimum sodium requirement for adults as 500 mg per day and the maximum salt intake as 6 g (2.4 g sodium) per day (WHO, 2012). Considering this amount, although the heavy metals in

salts do not pose a health hazard, care may be required in countries with high salt consumption.

Conclusion

It was concluded that salts consumed by humans contain different levels of minerals and heavy metals according to their types. Although exposure to heavy metals at recommended daily intake values do not seriously affect human health, they can lead to detrimental problems in the event of excessive consumption. It is also recommended that more research be carried out on salts consumed by people living in other countries.

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Chapter 15

FACIAL MIMIC MUSCLES: FUNCTIONAL ANATOMY AND VARIATIONS

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Introduction

Facial mimic muscles are located between the two leaves of the superficial fascia in the face area. They usually originate from the bone structures that make up the facial and head skeleton or from the fascia surrounding this area and insert at the skin (1, 2). These muscles play a role in the formation of various facial expressions such as joy, anger and fear. Mimic muscles are examined in five groups: scalp, auricular, eye, nose and mouth muscles. All muscles are innervated by the facial nerve (2).

1. Scalp Muscles

The epicranius muscle is divided into two parts, the occipitofrontalis and temporoparietalis muscle.

1.1. Occipitofrontalis Muscle

This flat leaf-shaped muscle, extending from the occipital bone posteriorly to the eyebrows in front, has two parts, the frontal and occipital bellies. A large aponeurotic structure called the galea aponeurotica connects these two parts in the middle. The frontal belly is four-sided like the occipital belly, but it is wider than it, the fibers are longer, thinner and lighter in color. Both ends of the frontal belly do not attach to bone. The inner parts of the muscles of both sides are continuous with the procerus muscle at the root of the nose. The middle fibers fuse with the corrugator supercilii muscle and the orbital part of the orbicularis oculi muscle. The outer fibers fuse only with the orbital part of the orbicularis oculi muscle. The muscle fuses superiorly with the galea aponeurotica near the coronal suture. The lower half of the inner part of the frontal belly merges with each other in the upper part of the nasal root. The occipital belly begins with a short tendon from the outer 2/3 of the superior nuchal line in the occipital bone and the mastoid part of the temporal bone. Muscle fibers that run parallel to the top end by attaching to the posterior end of the galea aponeurotica. There is a great deal of space between the muscles of both sides, where again an extension of the galea aponeurotica (3).

1.2. Temporoparietalis Muscle

This muscle, which is in the form of a wide and thin layer, was previously considered to be the auricle muscles. It originates from the temporal fascia in the anterior and upper part of the ear and inserts above the lateral margins of the galea aponeurotica. In addition to the two bellies of the occipitofrontalis muscle, it attaches to superior nuchal line and posteriorly to the external occipital protuberance. In the anterior, it extends to close the gap between the frontal hubs. The temporoparietalis muscles are attached to the sides. Here, the structure of the galea aponeurotica changes and be-

comes looser and extends as an areolar structure over the temporal fascia. Galea aponeurotica is tightly attached to the skin, but separated from the pericranium by a facial space. Therefore, galea aponeurotica can move on the pericranium together with the skin to which it is firmly attached.

When both bellies of the occipitofrontalis muscle work together, it pulls the scalp back, pulls the eyebrows up, creates wrinkles and grooves on the forehead skin. When the frontal belly works alone, it just pulls the eyebrows up. The temporoparietal muscle stretches the scalp by pulling it from the sides. Working together with the occipitofrontalis muscle, it contributes to the formation of grooves in the forehead and opening of the eyelids more. It also serves to slightly lift the auricle. The temporoparietal muscle and the frontal belly are innervated by the temporal branch of the facial nerve, while the occipital belly is innervated by the posterior auricular branch of the facial nerve. The frontal and occipital bellies show considerable variation in length and volume.

Sometimes it may be absent or the muscles of both sides may be fused to each other. When the frontal bellies fuse with each other, they can be seen as interlaced teeth at the fusion site. The occipital belly can sometimes fuse with the posterior auricular muscle. The transversus nuchae muscle (occipitalis minor muscle) can be seen as a thin strip of muscle in 25%. This muscle originates from the external occipital protuberance or the highest nuchal line (linea nuchalis suprema) and passes sometimes deep and sometimes over the surface of the trapezius muscle, mostly mixing with the posterior auricular muscle. Sometimes it inserts at the posterior margin of the sternocieidomastoid muscle.

2. Auricular Muscles

The anterior auricular muscle is a thin and inconspicuous muscle. It originates from the anterior part of the temporal fascia and inserts in the protrusion on the anterior side of the helix. Superior auricular muscle is slender, fan-shaped, originating from the galea aponeurotica and ending in a thin, flattened tendon above the root of the auricle. The posterior auricular muscle, which is in the form of two or three bundles, originates from the mastoid process via a short aponeurosis and inserts it in the posterior lower part of the root of the auricle (3). Advances in plastic surgery have demonstrated the potential benefit of this muscle in improving the results of otoplasty procedures (4). The muscle has recently been found to be a useful landmark for concho mastoid suture placement without disrupting its attachments (5). Agenesis of the posterior auricular muscle is associated with malposition of the auricular cartilage. In a study examining 99 right and left posterior auricular muscles, a single tendinous insertion was observed in most muscles (73.7%), two different muscle slips with a

single localized insertion in 23.2%, and triple insertion of three muscles on the concha (3.0%) (4).

Functionally, the anterior auricular muscle pulls the auricle anteriorly, the superior auricular muscle pulls the auricle upward, and the posterior auricular muscle pulls the auricle posteriorly. These muscles lose their importance in humans and work with the occipitofrontalis muscle rather than the ear to move the scalp. However, some people can move their auricles a little through these muscles. Innervation of the anterior and superior auricular muscles is provided by the temporal branch of the facial nerve. The posterior auricular muscle is innervated by the posterior auricular branch of the facial nerve (3).

3. Muscles Around the Eyes

3.1. Orbicularis Oculi Muscle

It has three parts, the palpebral, orbital, and lacrimal parts (Figure 1) (3, 6). There are solid connective tissue structures resembling a half moon called superior and inferior tarsus inside the eyelids. The outer ends of these tarsuses attach to the zygomatic bone via the lateral palpebral ligament, while their inner ends attach to the maxilla (frontal process) via the medial palpebral ligament. Superficial to the lateral palpebral ligament is the lateral palpebral raphe. The lateral palpebral raphe is composed of the fusion of the muscle fibers (palpebral part) coming from the lower and upper eyelids. Sometimes there may be some fat tissue or lobules of the lacrimal gland between the two structures. This thin part is pale in color, and is located within the eyelid. Fibers originating from the medial palpebral ligament terminate at the lateral palpebral raphe. The orbital part remains at the periphery of the palpebral part. The orbital part is thicker and darker in color, originating from the medial palpebral ligament, and laterally, it forms a ring without attaching to any place and ends in the same structure. The upper portion of the orbital part is fused with the frontal belly and corrugator supercilii. The lacrimal part is a small and thin muscle (average width 6 mm, length 12 mm), and is located posterior to the medial palpebral ligament and lacrimal sac. It emerges from the lacrimal bone (posterior lacrimal crest) and wraps the lacrimal sac first behind and then outside. It inserts in the medial ends of both tarsuses and medial palpebral ligament in the form of two strips above and below. Sometimes the pars lacrimalis may not be very prominent.

The orbicularis oculi muscle is the sphincter of the eyelids. As with blinking and sleep, the palpebral part allows the eyelids to close normally without effort. The orbital part ensures that the eyelids are tightly closed. When both parts of the muscle contract together, they pull the skin of the

forehead, temple and cheek a little medially, as well as tightly closing the eyelids. The antagonist of this muscle is the levator palpebrae superioris muscle. As the orbicularis oculi muscle closes the eyelid, the medial palpebral ligament is stretched. The lacrimal sac's wall attached to this ligament is pulled outward and anteriorly, creating negative pressure in the sac. This event causes the excess tears accumulated in the eye to be absorbed. The lacrimal part pulls the medial part of the eyelids back and presses the lacrimal sac, allowing the absorbed tears to descend downward (3). Its excessive contraction can cause a "crow's feet" rhythm at the lateral edge of the orbita (7).

The arteries supplying the orbicularis oculi muscle are the facial artery, branches of the superficial temporal artery, and the ophthalmic artery. The upper half of the muscle is innervated by the temporal branch of the facial nerve, but the lower half is innervated by the zygomatic branch. In conditions such as Bell's palsy, the eyelids cannot close well if the muscle cannot function. In benign essential blepharospasm, the muscle may contract involuntarily. This can cause serious difficulties in performing activities such as reading and driving (8).

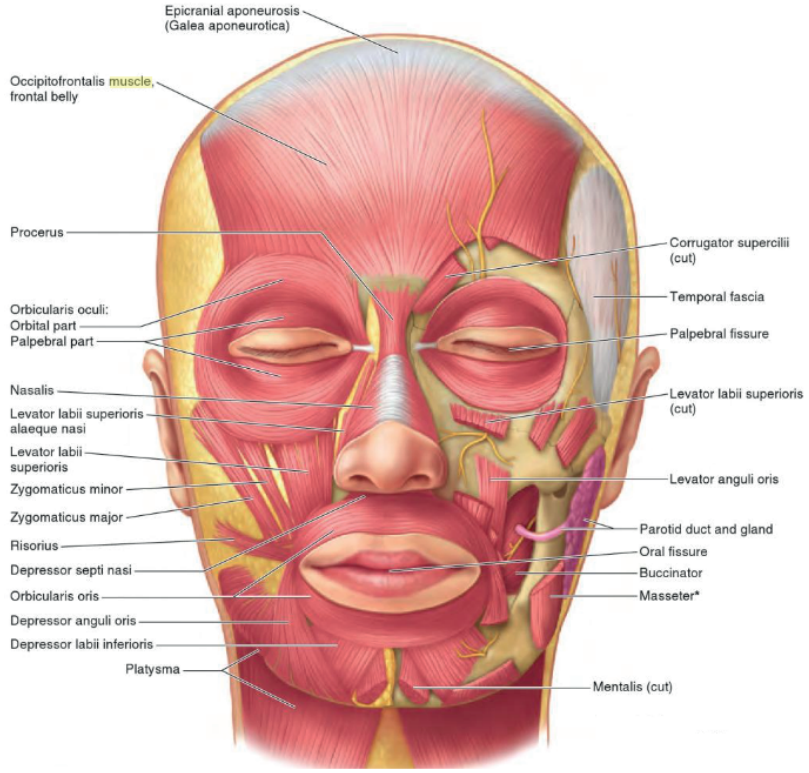

Figure 1. *Facial Mimic Muscles (6)*

3.2. Corrugator Supercilii Muscle

This muscle, which is small, thin and long according to general anatomy information in textbooks and literature, is located in the medial half of the eyebrows, deep in the orbicularis oculi muscle and frontal belly. The muscle emerges from the medial end of the superciliary arch. Extending upward and outward, it lies between the orbital and palpebral part of the orbicularis oculi muscle. It ends by attaching to the skin above the middle part of the superciliary arch (3).

Paired corrugator supercilii has two heads: transverse and oblique. The transverse head emerges from the superomedial edge of the orbital margin and serves to pull the eyebrows medially. The oblique head is smaller than the transverse head, runs parallel to the depressor supercilii muscle. It depresses the medial part of the eyebrow (9, 10). These movements cause vertical wrinkles on the supranasal skin of the forehead. They also create a frown expression when a person is angry or confused and act as mimetic muscles that form an important component of the "superficial musculoaponeurotic system" (SMAS) in the upper part of the face (9).

Variationally, in one study, the muscle was observed as three or four thin, rectangular, panel-like muscles. It was observed that the muscle had a larger mass than known, the base of the bone origin was quite wide, and it extended along the supraorbital notch/foramen at a distance of 0.6 cm from the midline. The muscle groups followed an oblique parallel course, but there were no distinguishable oblique or transverse components. These findings demonstrated a different outcome from previous studies to describe the corrugator supercilii muscle (11).

The lateral edge of the corrugator supercilii muscles may also show variability. In some individuals, the muscle may extend up to the lateral third of the eyebrow (9). In a study examining 30 separate articles (721 hemifaces), 28% of the muscle was classified as oblique and transverse head, and 72% was unclassified. According to other results of this study, the corrugator supercilii muscle emerged mostly from the medial supraorbital rim, followed by the medial frontal bone, the medial infraorbital rim, and the upper nasal process. In some cases, the corrugator supercilii muscle extended along the frontalis and orbicularis oculi muscle, while in some cases it extended either only along the frontalis muscle or only along the orbicularis oculi muscle. The muscle extended either superolaterally or laterally. Corrugators were most commonly embedded in the middle of the eyebrow or the middle half of the eyebrow, as well as in the glabella region. The length of the muscle ranged from 38 to 53 mm, and the transverse head was longer than the oblique head. The muscle was thicker in the medial canthus than in the mid-pupillary line. As a function,

the movement of pulling the eyebrow towards the medial was the most observed (91%). Medial brow lift and lateral brow depression were detected in 9% (12).

The muscle's arterial supply is supplied by the supraorbital and supratrochlear arteries of the ophthalmic artery (3). Neural innervation is provided by the temporal branch of the facial nerve (13). However, in the literature, while the temporal branch functions most in the innervation of the muscle, it has been stated that the zygomatic branch or angular nerve also innervates the corrugator supercilii muscle in some cases. They also emphasized that, as an important component of the glabellar complex, the corrugator supercilii muscles play an important role in maintaining the position of the eyebrow. Therefore, these anatomical variations should be taken into account during the surgical procedure (9, 12).

3.3. Depressor Supercilii Muscle

It is located on the medial side of the corrugator supercilii muscle and extends from the inner part of the palpebral part upwards to the brow skin (3). They may be variably fused with the surrounding muscles. There has been considerable speculation about the depressor supercilii muscle, particularly whether it is a different muscle or an extension or branch of the medial head of the orbital orbicularis oculi muscle (14). Based on the following, some authors have claimed that the muscle is a separate muscle in its own right. (I) In the study, each contained adipose tissue and neurovascular structures by different anatomic planes and was readily separated from the orbicularis oculi and corrugator supercilii muscles. (II) The fibers of the muscle were markedly superior in orientation and had a more reddish color, in contrast to the orbicularis oculi muscle. (III) The bony origin of the muscle was evident, the superiorly directed muscle belly was readily isolated, and its insertion in the medial brow skin was frankly visible (14, 15). In the study of Cook et al. in 18 cadaver hemifaces, it was observed that the depressor supercilii muscle emerged from the frontal process roughly 1 cm above the medial canthal tendon of the maxilla. As a new finding, the researchers revealed that in most samples, the muscle originated from two different heads (14). In another cadaver study (44 samples), it was observed that the depressor supercilii muscle was attached to the levator labii superioris alaeque nasi muscle or / and to the lower fibers of the orbicularis oris muscle by muscle fibers or thin aponeuroses, while in some samples there was no connection (16). The function of the depressor supercilii muscle is to pull down the medial parts of the eyebrows, and the muscle is innervated by the zygomatic and temporal branches of the facial nerve (3).

4. Nasal Muscles

4.1. Procerus Muscle

Procerus is a small pyramid-shaped muscle located at the root of the nose. It emerges from the lower part of the nasal bone and the adjacent nasal cartilage, and the muscle fibers terminate in the skin between the two eyebrows (3). It is located inferomedial to the bilateral frontalis muscles. It works together with other muscles of the glabellar complex (procerus, orbicularis oculi, frontalis, corrugator supercilii and depressor supercilii muscles) (17). The procerus muscle pulls down the medial parts of the eyebrows and creates wrinkles at the radix of the nose (3).

The muscle is supplied with blood from the branches of the facial artery, and is innervated by branches of the facial nerve. It is seen that the sources in the literature do not agree on the branches that innervate the muscle. In a study, it was found that the temporal, buccal and zygomatic branches of the facial nerve innervate this muscle (18). There are also studies where the angular nerve, which originates from both the zygomatic and buccal branches of the facial nerve, provides the primary innervation of the procerus muscle (19).

The procerus muscle has clinical importance in terms of structure, functionality, arterial nutrition and associated glabellar complex in surgical and cosmetic interventions such as injectable fillers, botulinum toxin injection and blepharoplasty. Disruption of the normal vasculature can cause tissue necrosis. In addition, disruption of the normal innervation of this muscle and the glabellar complex can cause facial sagging in the middle region of the eyebrow (20).

4.2. Nasalis Muscle

It consists of two parts; alar and transverse. The transverse part emerges from the outer-upper part of the incisive fossa, its fibers run upward and inward, ending in the middle with the fibers of the opposite side. Its upper fibers merge into the tendon of the procerus muscle (3). The upper transverse part on the back of the nose is called the compressor naris, and the lower alar part, which moves down on the sides of the nose, is called the dilator naris. It compresses the nasal aperture between nasal cavity and vestibule. The alar part originates from the lower portion of the maxilla, and medial to the maxilla towards the transverse part and attaches to the alar cartilage of the nose (21). The alar part widens the nostrils, while the transverse part narrows it (3). Its hyperactivity can cause oblique rhytides (bunny lines) of the upper lateral nose (21). Like all facial muscles, nasalis muscle is also innervated by the facial nerve (3).

4.3. Depressor Septi (Nasi) Muscle

It emerges from the maxilla above the central incisor and partially contains the fibers of the orbicularis oris. It extends upward to the columella to attach to the lower surface of the cartilage of the nasal septum. Its function is to pull the nasal septum downward (21). It's a well-known soft tissue structure in rhinoplasty that influences the final outcome (22). Variably, the size of this muscle may change or it may not be present (3). De Souza Pinto et al. performed the dissection of the muscle in 15 cadavers. The authors observed that the median portion of the muscle was attached to the distal portion of the Pitanguy's ligament and advised surgeons to consider this association with nasal ptosis (23). In another study examining 55 cadavers, the muscle originated entirely from the orbicularis oris muscle (62%). It was found that the muscle originated from both the orbicularis oris muscle and the maxillary periosteum (22%), and the rate of the muscle in its primitive form or absent was 16% (24). Conversely, Daniel et al. reported that the this muscle emerges only from the maxilla (25). Hwang et al. found that the muscle was inserted at the base and lateral surface of the medial crura. The authors suggested that the muscle is adjacent to the dermatocartilaginous ligament and that this connection is responsible for the end effect (26). Despite differences in anatomical reports on muscle, it is commonly thought that it originates from the maxilla and/or orbicularis oris muscle. More importantly, the muscle attaches to the medial crura and adjacent soft tissue. Disruption of this relationship forms the basis of the surgical treatment of tip descent during animation (22). Innervation is provided by the buccal branch of the facial nerve (3).

5. Muscles Around the Mouth

5.1. Levator Labii Superioris Muscle

Just above the infraorbital foramen and below the orbital margin, some fibers originate from the maxilla and some fibers originate from the zygomatic bone. Muscle fibers stretch downward and inward. In the upper lip, it ends between the levator anguli oris muscle and the levator labii superioris alaeque nasi muscle (3). Its contraction elevates the upper lip and deepens the nasolabial line. The buccal and zygomatic branches of the facial nerve innervate the muscle (21).

5.2. Levator Labii Superioris Alaeque Nasi Muscle

The fibers starting from the upper part of the frontal process extend downward and externally and divide into two parts. The inner part ends in the nasal cartilage and the skin, the outer part enters the upper lip and fuses with the levator labii superioris muscle.

In addition to elevating the upper lip, also widens the nostrils. Together with the levator labii superioris and zygomaticus minor muscles, it forms the nasolabial sulcus (3). The buccal and zygomatic branches of the facial nerve innervate the muscle (21).

5.3. Levator Anguli Oris Muscle

It is located in the deepest layer of mimic muscles together with the buccinator and mentalis muscles. The muscle originates in the canine fossa of the maxilla, approximately 1 cm below the infraorbital foramen. Its fibers extend anteroinferiorly to enter the modiolus of the oral commissure, and ends at the corner of the mouth. The buccinator, zygomaticus major, risorius, depressor anguli oris, levator anguli oris and orbicularis oris muscles contribute to the modiolus. Each muscle exerts a force on the oral commissure in a different vector, causing numerous variations and nuances of facial expression (27).

The primary function of the muscle is to elevate the corner of the mouth. It works in concert with the zygomaticus major muscle, which raises and lateralizes the oral commissure by moving it obliquely upwards and laterally. The different contraction rates between the zygomaticus major and levator anguli oris muscles help to reveal the unique features of an individual's smile (28). This muscle is very important because of its critical role in the formation of a natural-looking or "Duchenne" smile. Without this type of smile, people have difficulty expressing their feelings and maintaining human social connection (27).

The blood supply is provided by various small branches of the facial, internal maxillary and superficial temporal arteries. Its venous drainage predominantly flows into the facial vein, which empties into the internal jugular vein. The terminal buccal branch of the facial nerve innervates the muscle (28). Lymphatic fluid flows first to the preauricular, infra-auricular, parotid, nasolabial, buccinator, submandibular, submental, internal jugular, and anterior jugular lymph nodes and from there to the cervical lymph nodes (27).

5.4. Zygomaticus Major Muscle

Along with the orbicularis oris muscle, it is an important muscle in expressing happiness and joy. It emerges from the zygomatic bone in front of the zygomaticotemporal suture, extends downward and medially, and ends by fusing with other muscles at the corner of the mouth (29). Insertion of the zygomaticus major into the modiolar space can create fiber attachments to the orbicularis oris, levator anguli oris, buccinator, and depressor anguli oris muscles (30). The topographic position of the muscle is important for creating a healthy and natural smile restoration (29). It pulls

the lips superolaterally at an angle of 55.5° to the transverse plane to create a smile during facial animation. Zygomaticus major is often depicted as a single belly muscle and is an important facial expression muscle with a prevalence of 97% to 100% of individuals. However, the bifid zygomaticus major variant has been reported in the literature. The bifid zygomaticus major muscle is a known anatomical variation that clinically presents as a dimple in the cheek (31). Dimples occur in both sexes without special dominance. It can be bilateral or unilateral and is considered a congenital birth defect (32). The bifid variant begins with a single muscular belly of the zygomatic bone and progresses through the regular trajectory before dividing into two bundles, upper and lower, in the subzygomatic cavity. The superior bundle inserts into the usual place, while the inferior bundle enters the modiolus below the corner of the mouth (33). Since the inferior bundle usually has a dermal connection along the middle part, when the person smiles, the traction on the skin over the muscle creates a dimple due to the dermal tethering effect (34). This contributes to the understanding of the various facial muscle morphologies and attachment patterns that have important implications in surgical planning and procedures for facial rejuvenation and recreation of natural patient appearances.

In a meta-analysis study (259 cadavers) examining 7 cadaveric studies in different population groups, the prevalence of the bifid muscle was 22.7% (31). In addition, various zygomaticus major muscle shapes such as band-like, fan-like, rather than a singular form have also been identified (29). The zygomaticus major muscle originates from 5 to 6 cm anterior to the tragus. Its location has been recognised as a landmark for the main zygomatic and upper masseteric-retaining ligaments and the zygomatic branches of the facial nerve, which remain constant in the topographic anatomy (35). In addition, this muscle is the touchstone for the zygomatic branch of the facial nerve. Anatomically detailed knowledge of this muscle is very important for correct and appropriate intervention in trauma, tumor, face lift and parotid gland surgeries (29). Mowlavi et al. described the upper lateral margin of this muscle as a landmark for the safety of extended SMAS dissections in facelift (36).

Although it is stated in the books that the muscle is only innervated by the buccal branch (3), there are conflicting data on whether the buccal or zygomatic branches are responsible for the motor innervation. Although the literature does not provide a clear distinction about the transition region between these two branch, in the study of Kehrer et al, it was reported that the zygomaticus major muscle was innervated by zygomatic branches (67% of cases) and buccal branches (33%) (106 hemifaces) (37).

5.5. Zygomaticus Minor Muscle

It emerges from the outer surface of the zygomatic bone, behind the zygomaticomaxillary suture. In the upper lip, it ends between the levator labii superioris and zygomaticus major muscles (3). The muscle lifts the upper lip when smiling and exposes the maxillary teeth. In doing so, it works together with the zygomaticus major, the levator labii superioris and levator labii superioris alaeque nasi muscles (38, 39). Muscle is also involved in the formation of negative facial expressions, it aids to curl the upper lip while showing an expression of smugness and contempt. It also contributes to the deepening of the nasolabial sulcus in the expression of sadness (39).

The zygomaticus minor muscle varies in length and degree of fusion with neighboring muscles (3). Previous investigators have recorded cases where they could not find the muscle, and vague differences between these data have been reported. In Pessa's study, it was reported that the zygomaticus minor and major muscles looked very similar, could not be distinguished in the cases (34.4%), and the prevalence of the zygomaticus minor muscle was only 36% (40). Choi et al. found that the muscle attached only to the upper lip (63.0%), was attached to both the lateral alar region and the upper lip (27.8%), either absent or only undeveloped zygomaticus minor muscle fibers (9.2%) (39). In the study of Youn et al. (sixty-one hemifaces), the prevalence of cases in which the zygomaticus minor extends more towards the corner of the levator labii superioris muscle, not directly from the upper lip, was found to be 36.1%, since the lower border of the orbicularis oris muscle was covered by the origin of the zygomaticus minor muscle. The prevalence of mixing of the bellies of the orbital part of the outer edge of the orbicularis oris muscle with the zygomaticus minor was 88.5%, and the prevalence of mixing of the zygomaticus minor band with the lower inner corner of the orbicularis oris muscle was 55.7%. In the same study, the blending area of the orbicularis oris and zygomaticus minor muscles was located 17.8 mm below the Frankfurt plane. In addition, this area was located 8.9 mm lateral to the vertical line between the lateral canthus of the lateral portion of the orbicularis oris and the Frankfurt plane. In this position, the mixed umbilicus extended medially at a distance of 16 mm (38). In addition, Hamra stated that while dissecting under the medial parts of the zygomaticus minor and zygomaticus major muscles while performing a composite rhytidectomy, it is extremely difficult to separate the orbicularis oris and the zygomaticus minor muscles, resulting in edema (38, 41). These variant outcomes should be considered for understanding facial expressions and performing composite rhytidectomy. The buccal and zygomatic branches of the facial nerve innervate the muscle (21).

5.6. Risorius Muscle

It is a narrow bundle of muscle fibers originating from the fascia of the masseter muscle, extending horizontally anteriorly on the superficial platysma, and ending in the skin of the corner of the mouth (3, 42). Like other facial muscles, the risorius muscle has a higher percentage of slow-twitch muscle fibers than any other skeletal muscle in the body and contains a more complex configuration of the innervation of the extrafusal fibers (42). It is located deep within the SMAS. Its vertical septa connect the SMAS to the dermis (43).

Risorius muscle pulls the corners of the mouth to the side by contracting with an outward and upward movement, as it does during laughing, and helps to form facial expression (Figure 2) (3, 6, 42). Facial artery and transverse facial artery provide blood supply. Submandibular and mandibular lymph nodes receive lymph drainage from the risorius (42). The mandibular and buccal branches of the facial nerve provide innervation (3).

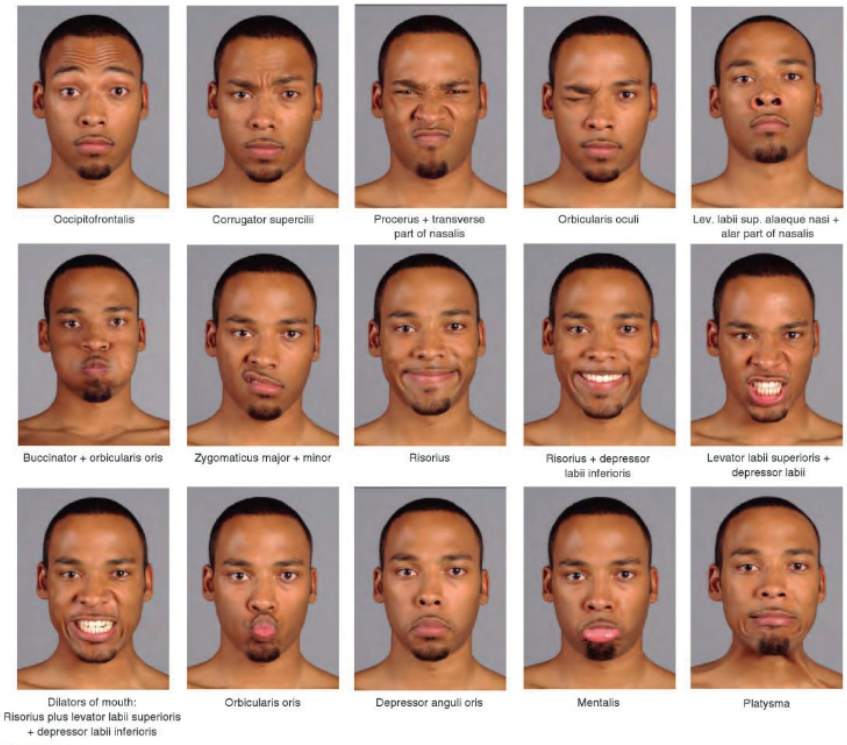

Figure 2. *Facial Expressions (6)*

Most variation is seen in the risorius muscle. This muscle, which is not of bone origin, is sometimes absent, sometimes double, sometimes fused with the large or platysma muscle (3). The anatomy of the risorius muscle is highly variable, as it can range from one or more thin fascicles

to a single broad muscle band. Bae et al. described different origins of this muscle from the SMAS layer, the parotid-maseteric fascia, or the masseter tendon. Some risorius muscle fibers may co-locate with the facial nerve and parotid canal in the deep facial plane of the face and other fibers may lie with the platysma muscle in the SMAS plane (44, 45). Another cadaveric study showed significant interindividual differences in bilateral symmetry, asymmetry, and presence or absence of the risorius muscle (46). In the literature, Germann et al. found that the muscle was located on both sides of the cheeks in most of the cases (risorius incidence 95.8%) (42).

Some authors have reported different arrangements of the risorius muscle. They reported that, depending on the insertion pattern of the risorius muscle in cadavers specific to the Asian population, there were three different types such as zygomaticus risorius, platysma risorius and triangularis risorius (47). They also revealed that these different types of risorius may allow different functions of the muscle. It may retract the labial commissures, or be part of the labial commissures, or may not have any elevating effect on the lips, or may even press the mouth angle downward to produce an expression of sadness. In the case study of Ferraira et al., the risorius muscle fibers on the surface of the masseter fascia were formed in two bands (45).

Since the SMAS layer is surgically dissected during aesthetic operations such as facelift and facial muscles are manipulated with surgical approaches, special care should be taken when performing dissections at the level of the parotid-maseteric region to prevent injury to the branches of the facial nerve, the masseteric ligament and the fibers of the risorius muscle (42). As can be seen, the risorius muscle is not an essential muscle, and its fibers are not always easily distinguishable, since it shows a high variability. It is important to have detailed knowledge of these different morphological arrangements of the muscle when planning and performing procedures (45).

5.7. Depressor Labii Inferioris Muscle

It is a small and quadrangular muscle. It originates between mental foramen and mental symphysis, extends upward and inward and ends by attaching to the lower lip skin. Here it fuses with the orbicularis oris muscle and the muscle of the opposite side. It is continuous with the platysma at the beginning. There is plenty of adipose tissue between its fibers (3). In the origin part of the muscle, the most lateral 1-2 cm part is covered by the origin of the depressor anguli oris muscle. From its origin up to 3 cm wide, the muscle fibers are directed cephalad and medially, covering the mental nerve located on the deep surface of the middle part of the muscle. The fibers insertion on the superficial and inferior surface of the orbicu-

laris oris, and intertwine with this surface. Therefore, there are fibro-muscular attachments to the skin and vermilion of the lower lip. Its insertion into the middle portion of each half lip is almost 2 cm wide.

The function of the muscle is to rotate the vermilion outward, pull the lower lip down and move it laterally. In marginal mandibular nerve palsies, paralysis of this muscle is the major reason of the deformity. The location of the muscle can vary from person to person. The position can be easily determened by asking the patient to show their lower teeth. The lower lip can be palpated along the vermilion border by gently pushing the lip upwards. The middle and lateral parts of the lip can be easily pushed upwards with a finger, but resistance is encountered in the upward and medial movement between the medial and lateral 1/3 of the lip. The location and width of the muscle belly can be determined by the location of this resistance.

The marginal mandibular nerve innervates the lower lip muscles. Paralysis of the nerve is an aesthetic problem and may occur in isolation or as part of a unilateral facial nerve palsy. Isolated paralysis of the nerve may be congenital or may result from nerve trauma. The nerve can be damaged during surgical interventions such as neck dissection, meloplasty, and submandibular gland resection. The nerve usually consists of two to three terminal branches of the facial nerve. Secondary repair of the nerve is difficult and its consequences are unpredictable. After free muscle transfer for smile reconstruction in unilateral facial paralysis, the most obvious deficiency in smile is usually the asymmetry of the lower lip due to lack of depressive function (48).

5.8. Depressor Anguli Oris Muscle

It originates from the anterior part of the lower edge of the body of the mandible, extends upwards and ends at the mouth corner. Its fibers are continuous with the platysma muscle at the beginning and with the fibers of the orbicularis oris and risorius muscles at the ending point. Some of its fibers continue with the fibers of the levator anguli oris muscle. It pulls the corner of the mouth downward as an antagonist of the zygomaticus and the levator anguli oris muscles. When the depressor anguli oris muscle works together with the levator anguli oris muscle, it pulls the corner of the mouth medially (3). Its contraction depresses the angle of the lower lip, causing the mouth to open. Increased use can cause radially oriented lower lip rhytids, known as "marionette lines" (7). It can be seen as two or three separate strips as variations. The buccal and mandibular branches of the facial nerve innervate the muscle (3).

5.9. Mentalis Muscle

It originates from the alveolar juga of the lower lateral incisors, extends downward and inward and inserts at the skin of the chin. The mentalis muscle pulls the lower lip up and extends it forward and creases the chin. In the meantime, a groove forms in the skin of the chin (3). Its overuse may be responsible from a "poppy chin" pincushioning effect on the mentum (7). The mandibular and buccal branches of the facial nerve innervate the muscle. The size of the mental muscle and the degree of fusion with the platysma muscle can be highly variable (3).

5.10. Transversus Menti Muscle

The depressor muscles of the mouth, which are part of the superficial facial muscles, are important in the evaluation of some cosmetic or reconstructive facial plastic surgeries. The transversus menti muscle is also a superficial muscle located in the submental region and is clinically accepted to be used in the reconstruction of some defects around the mouth angle (49). It is known as a muscle strip originating from the depressor anguli oris muscle, crossing the midline at the tip of the chin and passing as transverse fibers to the opposite muscle (50). This small muscle may be absent in 40% (3). Its function is to depress the mouth angle and shift the angle towards the medial side (50).

Because the muscle extends transversely between the left and right depressor anguli oris muscles along the jaw in the adult, its myotomes may differ from the same myotomes of the depressor anguli oris muscle. Therefore, although this muscle is considered a variant type of the platysma muscle, it should also be classified as a variation of the depressor anguli oris muscle (51). Nowadays, cosmetic or reconstructive facial plastic surgeries, including submental area rejuvenation, are relatively popular. In the rejuvenation of the submental region during cervicofacial rhytidectomy, the difficulty level and limitation of the operation depend on the variant anatomy of the structures such as skin, subcutaneous fat, platysma muscle, etc. (50). It has been reported that the transversus menti muscle can be used for the reconstruction of some structures such as deformities around the mouth angle (49). The mandibular and buccal branches of the facial nerve innervate the muscle (3). Its blood supply is provided by small branches of the submental artery (50).

5.11. Orbicularis Oris Muscle

The orbicularis oris muscle is attached to the dermis of the upper and lower lip via a thin SMAS and is a complex, multilayered muscle that is an attachment site for many other facial muscles around the oral region (52). Some of the muscle fibers belong to the extensions of other mimic

muscles, and some of them belong to the fibers in the main lip (3). Neither bone nor tendon has an origin. Its deep fibers originate from the modiolus on both sides. These fibers pass horizontally along the midline from one commissure of the oral cavity to the other and lie close to the inner mucosal surface. The lower edge of the fibers folds over itself, turning the mucous membrane over and forming the vermilion. The deep fiber acts as a constrictor and is responsible for the sphincteric action of the mouth. It is concerned with holding food due to general sphincteric activity with other muscle rings of the oropharynx; this is known as the "archaic" part of the muscle (53). Superficial muscle fibers originate from facial expression muscles and are divided into two bundles, upper and lower. The lower bundle (nasolabial) receives fibers from the depressor anguli oris muscle on both sides. They insert at the skin forming the ridges at philtrum with short fibers ending in the ipsilateral ridge and long fibers crossing the midline to insert into the contralateral one. The upper bundle (nasal) represents the common insertion site of fibers of various muscles such as the levator labii superioris, levator labii superioris alaeque nasi, transversus nasi, zygomaticus major and minor muscles. They enter the nasal spine (anterior) and the septo-premaxillary ligament from the nostril threshold and descend deep into the alar base (53, 54). The superficial fiber is the retractor fiber and is related to the facial expression needed during speech and the delicate movements of the lips (53).

Orbicularis oris is the main muscle that closes the lips. The fibers coming from the periphery and extending deeply and the main lip fibers with an oblique course firmly press the lips to the jawbones. Superficial fibers, especially those that cross, bring the lips closer together and pull them anteriorly (3). The muscle also works with the cheek muscles to apply pressure to the dental arches, creating contact between the teeth and lips and collaborating with them in the production of speech sounds (54 - 56). The muscle is also significant for swallowing, chewing and sucking movements (54, 57). Previously, the muscle was considered the sphincter muscle, as it surrounded the oral cavity. However, this theory is no longer widely accepted, as research has found that the fibers of this muscle run in different directions rather than running in a uniform circular shape.

As a variation, some newborns may be born with the absence of the orbicularis oris muscle on one side of the face, resulting in a partial droop on the affected side. In Bell's palsy, drooping of the orbicularis oris causes drooling (54). Decreased muscle tone causes the commissure to fall, which can cause drooling and difficulty eating (58).

The orbicularis oris muscle, together with the buccinator and pharyngeal constrictor muscles, has an important role in orofacial function (swallowing, sucking, whistling, chewing, pronunciation of vowels, kiss-

ing) known as the "buccinator mechanism" (59). If the buccinator and orbicularis oris muscles are weakened or paralyzed, food tends to accumulate at the mouth opening during chewing. Tapping the side of the nose or upper lip of infants causes elevation of the oral commissure on the ipsilateral side. This maneuver is called the orbicularis oris reflex (nasal reflex). This reflex disappears spontaneously in the later stages of lifetime (60).

The mental and infraorbital branches of the maxillary artery and the superior labial and inferior labial branches of the facial artery supply blood to the muscle (54). In addition, the superficial temporal artery supplies blood to this muscle through its transverse facial branch. Innervation is provided by the buccal and mandibular branches of the facial nerve (61).

Tightness in the muscle results in a smoker's upper and lower lip line or vertical lip line/rhytids (54). Contraction of the muscle results in marionette lines in the lateral perioral region. These lines give the illusion of sadness. The most critical structure affected in a cleft lip is the orbicularis oris muscle. Its correct direction is crucial for its normal function. During reconstruction surgery in cases of cleft lip, restoration of the anatomical and functional components of the upper lip is the primary objective that is imperative to restore the normal course of this muscle (53).

5.12. Buccinator Muscle

It is the main muscle of the cheek and is located lateral to the oral cavity. This quadrangular muscle is located deep to the other facial muscles and between the maxilla and mandible. It emerges from the alveolar process (maxilla) and pterygomandibular raphe of mandible along the three molar teeth. Constrictor pharyngis superior muscle attaches to the posterior side of this raphe. The upper and lower fibers, which gathers a little towards the front, extend without decussation, forming a deep layer on the lips at their own level. The middle part of fibers decussate. The fibers from below lie on the upper lip, and the fibers from above on the lower lip. The buccinator muscle is covered with a fat layer called buccopharyngeal fascia and corpus adiposum buccae, and its inner surface is covered with buccal glands and oral mucosa. The muscle is pierced by the parotid duct at the level of the upper second molar tooth.

The buccinator muscle pushes the food between the teeth by pressing the cheek against the teeth. That's why, it is considered an auxiliary mastication muscle. It functions to forcefully expel the air trapped in the mouth. That' why, it is also well-known as the blowing muscle (3). It plays an active role with the orbicularis oris and superior constrictor muscles during swallowing, chewing, blowing and sucking. One of the first muscles to act in a baby during sucking is the buccinator muscle (62). There are theories that the muscle thickens the buccal mucosa by acting as a

hydrostat. Because of this thickening, the cheek pushes the bolus against the tongue (63). When the muscle contracts, it also pulls the corner of the mouth to the side. The muscle contracts slowly when closing the mouth, and relaxes during the opening movement. This mechanism maintains the tension of the cheeks, thus preventing injury to the buccal mucosa (64). The buccinator muscle tends to move during the oral and pharyngeal phases of swallowing. Together with the orbicularis oris muscle, it creates a peristaltic wave-like contraction and initiates the swallowing movement in the oral phase. This wave passes through the pharynx. The buccinator muscle initiates the wave and orbicularis oris muscle quickly follows it. Apart from that, the buccinator muscle is activated during some mandible movements such as protrusion and retrusion. It does not directly cause these actions but assists the effort to perform them. As a physiological variant, although a rare phenomenon, the crestal attachment of the buccinator muscle may lead to difficulty in routine oral functions and restoring the edentulous area (62). The buccal branch of the facial nerve innervates the muscle (21).

6. Platysma Muscle

The development of the platysma begins with the SMAS at the 9th and 10th weeks of pregnancy, originating from the cervical lamina. At week 17, the platysma can be identified at the insertion point on the mandible (65). It emerges from the deep fascia covering the deltoideus and pectoralis major muscles. This thin, leaf-shaped muscle passes in front of the clavicle and extends upward and inward. The anterior segment fibers fuse with the same fibers of the opposite side in the lower and posterior part of the symphysis menti. Some of the posterior fibers end in the mandible (below the oblique line), while the other ends in the skin and subcutaneous connective tissue of the lower half of the face. Some of its fibers fuse with the fibers of the mimic muscles in the corner of the mouth (3).

When the muscle contracts, the concavity between the chin and the neck side decreases and tense oblique ridges form in the neck skin. The platysma pulls the lower jaw down. Thanks to its labial and modiolar connections, it can pull the lower lip and corners of the mouth outward and downward in facial expressions such as fear or surprise (66).

Platysma muscle is sometimes absent as variation, and sometimes it is seen as very thin or sparse fibers. It has been observed that it may end at the lower end of the chin, as well as extend up to the ear above (3). Very rarely, the platysma muscle fibers may extend as high as the zygomaticus muscle or may be seen at the margins of the orbicularis oris muscles (67).

In the study of Hwang et al. in which all anatomical studies on the platysma muscle were analyzed (443 hemifaces), the muscle by a majority emerged from the upper part of the thorax and anterior of the clavicle (67.7%). This was followed by the subcutaneous tissue of the acromial and subclavicular regions with a rate of 22.6% and the pectoral region with a rate of 9.7%. It was observed that the elevation of the platysma upwards and medially was 68.5%, and its elevation from the clavicle towards the face was 31.5%. In the cases, it was recorded that the muscle most commonly ended in the cheek skin with a rate of 57.5%. It has also been reported that it ends in the cutaneous muscles around the mouth, the mandibular cutaneous ligament or the zygom, and the parotid fascia or periosteum of the mandible. Although some sources indicate that the platysma is innervated only by the cervical branch of the facial nerve (3), in this review, the muscle was most commonly innervated by the cervical and mandibular branches of the facial nerve (60.5%) or cervical branch of the facial nerve (38.2%). In these cases, the most common movement of the muscle was to pull the lips down (83.3%) or back (12.9%) (186 hemifaces). The most common age-related change in the platysma was shortening (70.7%), followed by thinning (25.2%) (443 hemifaces). As structures close to the platysma, the mandibular branch was consistently found posterior to the platysma (100%) (180 hemifaces) (68).

The blood supply to the platysma comes from the branches of the external carotid artery. Even a minor trauma can cause bruising, as the muscle has an abundant blood supply. During surgery, hemostasis of the platysma layer is important before it progresses. Otherwise, continuous bleeding may close the surgical field. The lymphatic drainage of the muscle is carried out by the superficial lymph nodes of the corresponding soft tissue and is irregular, as it occupies the entire distance of the neck and parts of the face (67).

Conclusion

Insufficient facial animation and unsymmetrical facial expressions are conditions that require surgical or aesthetic intervention. Having a detailed knowledge of the functional anatomy of facial expression muscles is very important in terms of providing all treatment options and managing possible complications in a situation such as facial paralysis that deeply affects the patient psychologically, aesthetically and functionally.

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Chapter 16

REFLECTIONS OF THE PHYSICIAN'S OATH IN THE COVID-19 PANDEMIC

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Introduction

It is thought that Hippocrates carried out the oath of the profession of medicine in a village in Anatolia about 2500 years ago. After a long silence, the oath was used in ceremonies by scientists in Western medicine in the Middle Ages, and it was translated into many languages in the following centuries, allowing it to be read at the graduations of new graduates in medical faculties. (Frush, Eberly& Gross, 2018; İlgili et al.,2016; Antoniou et al.,2010; Yapijakis, 2009; Şahinoğlu,1994).

It has been an important question for centuries how the physician's stance should be towards society. (Antoniou et al.,2010). Young graduates are probably trying to answer this question in different ways by repeating in front of the public what moral rules they will follow with the medical oath. The medical oath is not mandatory; although it is not included in legal texts, it has a place in special regulations in medical faculties. The statements in the oaths that have gone down in history give us clues about the professional stance of the physician. In oath statements, there are moral sanctions and the rules to be followed in physicians' medical practices. Every expression in the oath has been shaped according to the time and geography in which it was found. (İlgili et al.,2016; Şahinoğlu,1994; Friedenwald,1917; Encyclopedia of Bioethics, 2004).

The most popular of the oaths is named after Hippocrates, but in the history of medicine, there are prayers, oaths, beliefs, institutional directives that guide health workers in many civilizations. If a few examples of oaths are given from history, which comes to mind at first is the text written by Imhotep in approximately 3000 BC in the Ancient Egyptian civilization, in which physicians stated that they would not misuse their power against their patients, and they will be aware of the needs of the society and fulfill their responsibilities. Also, the reference to sisters in the last statement of the Imhotep oath may be a warning of the existence of female physicians (Pickett,1992).

Charaka Samhita, one of the main texts of Ayurveda ("life science") in ancient Indian, urges medical students to self-sacrifice and devotion to duty, and in the text written by the Taoist writer Sun Szu-Miao in ancient China, the importance of preserving life and serving the interests of the patient was emphasized. (Friedenwald,1917; Menon& Haberman, 1970).The most important Jewish physician of the Middle Ages, Moses Maimonides (Ibn Maimonides, 1135-1204) expresses the importance of being able to practice the art of medicine with compassion and without the greed for fame and glory in the "Daily Prayer Of A Physician. (Friedenwald,1917).

The existence of Hippocrates and oaths has been seen in the Islamic world since the IXth century. In the Arabic oath taking place in Ibn Ebî Usaybi'a's (d. 1269) work called 'Uyûnu'l-Enbâ fî Tabakâti'l-Etibbâ, it is mentioned how the patient should be treated, and privacy should be respected.While the gods were witnessed in the physician's oath, in the Islamic world, the text of the oath was adhered to in the intervening centuries, but it was accepted that the healer was Allah and talented physicians were friends of Allah(İlgili et al.,2016).

While the prohibition of abortion and the prohibitions related to surgical procedures were noted in the first texts, including the oaths in Islamic medicine, statements suggesting that human dignity should be kept at a high level, such as autonomy and privacy, were added along with medical practices in line with the increase in modern technology and knowledge. (İlgili et al.,2016; Friedenwald,1917; Kao&Parsi,2004; Hanson,2004; Hajar,2017). It is noteworthy that the oaths after establishing the Republic of Turkey did not include expressions specific to faith(İlgili et al.,2016). Human dignity, patient rights, and non-discrimination come to the fore in the physician's promise(Gamble, 2019).

Today, oaths in medical faculties in Turkey are mainly similar to the physician's oath, the Declaration of Geneva, and the Oath of Professional Allegiance(İlgili et al.,2016; TMA, 2017; Packianathan et al.,2020). At the October 2017 general assembly meeting of the World Medical Association, the working group formed by the Turkish Medical Association and the medical associations of India, Germany, Sweden, the USA, India, and Israel updated the Declaration of Geneva written in 1948. In addition, with the suggestion of the Turkish Medical Association, WMA named the Declaration of Geneva as a sub-title "Physician's oath". It has been translated into Turkish by the Turkish Medical Association, and its use has been recommended to all medical faculties. The text of the oath read at Mersin University Faculty of Medicine is the "Physician's oath" recommended by the World Medical Association(TMA, 2017).

The general acceptance from past to present is that young graduates take the oath in a ceremony with their families and teachers. However, the type of pneumonia reported in China on the last day of 2019 was identified as a new coronavirus in January 2020. Covid-19 spread worldwide within three months and was declared a pandemic by the World Health Organization on March 12. In the second week of March, the first case was identified in Turkey, and face-to-face training was suspended as soon as possible with restrictions in many areas such as transportation, food, tourism, and work to prevent the spread of the disease. (Turkey's Response To Covid-19,2020).

Medical school education is also one of the restricted educations. Since medical education consists of two stages, theoretical and clinical, it can be argued that pandemic restrictions will affect students in two dimensions. First, basic theoretical knowledge and supportive practical applications were included, and they stayed away from the laboratory environment. In the second stage, the theoretical course and the practice at the bedside are carried out, and they are away from the environment in the patient-physician relationship. Many variables of physician candidates affect their academic success and are reflected in the patient-physician relationship while gaining professional skills, but it is difficult to comment on the reflections of pandemic restrictions. (Ögenler&Selvi,2014; Hueston, 2020; Seetan et al.,2021).

The distance education of medical students carries uncertainties regarding how it will affect their future and the patient-physician relationship. In the 2019-2020 academic year, sixth-grade physician candidates continued their internship applications online starting March 2020. Due to the pandemic restrictions, one step before the graduation of the medical faculty students, it has caused them to take the physician's oath to the agenda in a different way from other variables related to education and how the graduation ceremony will be.

Due to pandemic restrictions in Turkey in 2020, it has been decided not to hold graduation ceremonies in faculties. In this period, professional candidates and healthcare professionals are going through a significant test in terms of ethics as well as clinical sciences. Covid-19, a significant health problem that affects the whole world, affects the lives of all professional candidates and healthcare professionals in every way. Physicians, in particular, are faced with many dilemmas specific to professional ethics taking place in the physician's oath. (Seetan et al.,2021; Godlee, 2020).

In the midst of such dilemmas, it is of particular importance to read the oath regarding the professional belonging of newly graduated students. In this context, the study aims to determine the perspectives of newly graduated medical students about the medical profession oath to understand how they construct the meaning of the oath and social reality in their natural environment. In this way, the phenomenon of professional oath will be described in-depth and in detail for newly graduated physicians, and the complex relationship between the oath and their perspectives on the profession will be interpreted in its context.

Method:

Mersin University Faculty of Medicine 2020 sixth grade students requested that they take the physician's oath in small groups following the rules of pandemic restrictions. In Mersin University Faculty of Medicine,

face-to-face and online oath ceremonies were held for newly graduated students between May 2020 and June 2020.

In the 2020 academic year, 145 physicians who were entitled to graduate from the Faculty of Medicine and participated in the swearing-in ceremonies were included in the study. Our study used observations during the ceremonies held between 01 May and 01 July 2020. A standard data collection tool such as an observation chart was not used. Data from the participants' behavior and their study of the surrounding conditions were evaluated and interpreted. The researchers themselves took part in interacting with the students in the sample at the swearing-in ceremony. At each swearing-in ceremony, students were directly interviewed, interacted with, and empathized. Some situations were tried to be evaluated from the eyes of the students. The experiences during the oath were also experienced by the researchers. The experiences gained were used in the process of students' perspectives, data analysis, and interpretation. The inclusion criterion is that the students are Mersin University Faculty of Medicine graduates in the 2019-2020 academic year and attend swearing-in ceremonies (online or face-to-face). Being a graduate of Mersin University Faculty of Medicine in 2019-2020 but not attending the swearing-in ceremony and not being a graduate of this academic year are exclusion criteria.

Since a standard data collection tool was not used in this study, descriptive statistics about students were used, but the data were not reduced to numerical indicators. In our study, researchers are subjective, but the data obtained were expressed impartially and without prejudice, reflecting its richness, depth, diversity, and detail.

How did Mersin University Medical Faculty students read the Physician's oath?

In the past years, when graduating from medical school, the graduation ceremony of the students was held in the form of a big show. Months before the ceremony, all students who wanted to attend the ceremony were preparing by rehearsing with the advisor and technical service. However, the pandemic restrictions deprived students of this beauty. Since there was no graduation ceremony, the students asked the Faculty of Medicine, Department of History of Medicine, and Ethics for their opinion on the reading of the physician's oath. An academic board decision was taken by the Department of Medical History and Ethics, Mersin University Faculty of Medicine, to have the physician's oath read by the graduates. Based on this academic board decision, it was stated that the physician's oath could be read in groups following the pandemic restrictions. A graduation working group was formed, consisting of three newly graduated students, three

faculty members, and a person in charge of student affairs. Participants were informed by the working group that a maximum of ten people could participate in the oath reading and that there were no families. Faculty of Medicine Türkan Saylan meeting hall and the entrance floor of the dean's building were designated as the swearing-in place. The ceremony of the first three students was held with the participation of the Mersin University Rector. Other recent graduates honored the oath being read in small groups.

Following the pandemic restrictions, online oath readings took place via WhatsApp at the places they wanted (most of them were in their own homes). Those who had the oath ceremony online had one or two participants. Students' being present at the ceremony on time, participating as a group, preparing their clothes especially, and behaving following the pandemic rules are examples of positive desired behaviors. Despite the absence of an audience, all the readings took place in a ceremonial, emotional but semi-formal way. Although some of the students lived in different cities, they came to Mersin for the ceremony. Students expressed their gratitude as nearly 40 repeat ceremonies were held during the pandemic restrictions. During the ceremony, they listened carefully to the directions; the students stood at least one and a half meters apart and did not remove their masks except during reading. For each student, a printout of the oath was given to the students before the oath. They repeated all statements after the lecturer in a synchronized manner without errors. After the oath, the printouts were signed by the lecturer (researcher), who made the oath read.

RESULTS

In the 2019-2020 academic year, 222 students were entitled to graduate. 145 (65.3%) of the students attended the swearing-in ceremonies, while 75 (33.7%) did not. Of the participants in the swearing-in ceremony, 75 (51.7%) were female, 70 (48.3%) were male, 134 92.4% were graduates, 11 7.6% were still interns. 103 (71%) of the participants were in Mersin, 30 (20.6%) came to Mersin for the swearing-in ceremony even though they were outside of Mersin, 12 (8.2%) were outside of Mersin and participated online. One participant had an operation and was hospitalized, and the ceremony was held in the hospital.

Some of the opinions of the attendees about the physician’s oath:

“Written response to my conscientious obligation to my profession”,

“It made me feel that I started my medical profession and graduated”,

"It brings all my colleagues together on a common ground, but more lessons should be taught on this subject so that the ethical value can be better understood",

" Confidence is instilled at the beginning of the medical profession, and it completes us",

"I was very upset that our graduation coincided with a process like a corona. I was going to graduate from medical school once in my life, and I would like my family to experience this pride, but it did not happen; thanks to you, at least I took my physician's oath, thank you, I am glad you exist.",

"It was the physician's oath for me, a symbol of how indiscriminate and unlabeled my profession should be, as people all over the world classify and separate those around them for many reasons"A traditional oath",

"Value reminding me of my duties and responsibilities at the beginning of my professional life",

"An unwritten rule of medicine and respect for the profession",

"We must first respect our profession ourselves so that we can expect respect from other people as well",

"The first indication that we started our career",

"An emotional oath that makes our family and us proud",

"One of the cornerstones of medicine".

Some of the opinions of the participants about the swearing-in ceremony:

"Even though we cannot be in the same environment, I feel very lucky that it removed the distances and gave us the chance to read the physician's oath. I received very positive feedback about this application from my friends in many faculties",

"I never thought of not participating when I took the risk of organizing so many people, putting effort and dealing with them.",

"It was very valuable to me",

"Well thought out and implemented",

"It was done well in this short time, but it can be improved in the coming years",

"Made me proud".

"Thanks, now I am working as a doctor and thanks to you I am not sad".

DISCUSSION

In our study, it is a favorable situation that more than half of the new graduates participated in the swearing-in ceremony. Remarkably, they paid the utmost attention to the requirements of the ceremony without public ceremony, especially without their families and other faculty members. For centuries, it has been a ceremonial ritual for graduates to publicly reiterate that they will act following their professional competence and moral standards in oaths accepted as a social contract. Oaths take place in the form of ceremonies with the public's participation in many countries of the world as well as in Turkey or medical faculties in North America. The public ceremony reflects both individual and public perceptions of current medical practice(İlgili et al.,2016; Cruess & Cruess, 2014; Helmich E & Carvalho-Filho 2018). However, the reading of oaths by the participants in our study without third parties can be considered a stance against the idea of realizing the contract in front of the public in the literature. However, although it is not compulsory, the fact that the participants are very enthusiastic and eager to read the oath and the atmosphere is emotionally specific can be considered an indicator of the value of the oath. It can be argued that the physician's oath has a meaning independent of third parties other than the one who took the oath and the one who administered the oath. The technological development of people in the 21st century has paved the way for many changes in human interaction in today's conditions. (Prabhumoye et al., 2021; Körner & Volk,2014).

While the gods took part in the oath in ancient times, human dignity, absolute accuracy, and trust have come to the fore in oaths since the fifth century BC. The existence of the oath is accepted as evidence in societies. In monotheistic religions, for example, in Islam, the oath of two witnesses or an individual is considered sufficient to prove the veracity of an event. The Bible similarly forbids perjury and false oath. The person accepts to be punished when not fulfilling the oath; the inner compulsion of the oath is crueler than any bodily torture (Silving, 1959).

Although the professional oath of the participants is not legal or religious, the expressions in the content of the oath indicate that they accept a sanction against them. While the participants promise themselves about their profession, the lecturer performs the administering duty of taking the oath and witnessing. Although there was no public participation, the graduates fulfilled all the requirements of the oath. Participants' oath includes statements that reflect measurable contemporary ethics and laws. Action is invaluable given the role of oaths in promoting accountability of physicians(Hajar, 2017; Loewy,2007).

The similarity of the members in a professional group, their common interests and abilities specific to material, moral, and social requirements, the communication and interaction among the members, the logic of the formation of the group due to necessity or coincidence, and its continuity are the characteristics that make up the group. It is interesting that while occupational groups have different purposes and functions, they show similarities in countries at different times. However, it is clear that the professional group members will be affected by their culture (Werbel & Johnson, 2001; Çiçek et al., 2018).

While the oath has been talking about the moral relations between the patient and the physician and between the physician and their educators throughout history, the current oath includes social responsibility and rules that must be followed for all humanity. The dependence of medicine on science and technology and the economy's importance in practice are included in the social contract. Social expectations, moral responsibilities of physicians, legal mechanisms change over time. The mentality of physicians is adaptable to change over the centuries. (Cruess &Cruess 2014) In our study, the participants stated that they have a positive opinion about the moral rules in the oath. It is gratifying that their awareness of their duties, professional duties, and responsibilities towards humanity is included in the statements.

Hippocrates is a respected, human-loving, competent physician who adopts being helpful to the patient as a fundamental principle, keeps the patient's secret, is moral, has manual dexterity, is cautious not to harm the patient, observes nature, purifies medicine from religious elements, and bases it on reason and science. Although physicians before him are known, all the features attributed to his personality caused him to be accepted as the ancestor of medicine. Today, it is thought that these personal characteristics, which are taken as an example by physicians, and the desire to resemble the master of medicine are different from having mythological features. Medical historians cannot claim that any of the features attributed to Hippocrates are supernatural; each one of them, as a physician, are desirable characteristics in a role model(Kao& Parsi, 2004; Hanson, 2004; Yapijakis 2009).

Throughout history, Hippocrates' model personality makes his presence felt in the expressions in the oath. It is a positive feature in showing their acceptance of the features that the participants were asked to include in their statements during the swearing-in ceremony. The fact that the demand for action comes from the students in our study is proof of the breadth of the vision of young minds.

The modern physician's oath is a statement about how the newly graduated physician should practice medicine fairly and ethically and is generally held ceremonially. In Mersin University, as in many medical faculties worldwide, white coat ceremonies, stethoscopes, cadaver thanksgiving ceremonies are held(Hulkower,2016).

Due to the restrictions during the pandemic period, all crowded ceremonies have been canceled. The reading of the oath at the graduation ceremony or in a ceremonial atmosphere corresponds to the 18th century. However, in our study, an oath-reading ceremony was held independent of the graduation ceremony. Completing the profession, symbols, and self-confidence expressions may prove that physicians have internalized the oath. There are many criticisms, such as that the physician's oath is weakened in content and does not include medical practice, social or legal responsibilities, research ethics, and patient rights(Hulkower,2016).

However, it is certain that Hippocrates continues to influence physicians. It can be put forward as evidence that the new graduates performed the ceremony independently of religion, politics, and legal obligations and stated that they were proud of it. In the literature, it is an indicator similar to the specific structure of the oath to the profession of medicine(Hulkower,2016; Cruess& Cruess, 2014).

The oath ceremonies took place before the graduation date in the past, but it coincided with the beginning of the medical profession during the pandemic period. Ninety percent of the students participated in the swearing-in ceremonies after qualifying for the diploma. Considering Turkey's geographical and socio-cultural structure, the significance of the oath for the medical profession is revealed. The Physician's Oath represents patient-centered care, emphasizes communication, and opposes dogmas, religion, and magic by giving responsibility to the physician can explain its reaching beyond history(Hulkower,2016). It is thought that the fact that the text's content represents scientificity and moral values is influential in its adoption by the participants.

Taking the oath in medical schools has become common throughout the world in the twentieth century, but it is argued that the contents are different. The oaths provide information about the ethical values in the environments in which they take place(Gamble et al., 2019).

When we look at the content of the oath, there are differences in medical education and practice from the earliest times to the present. In their statements, the participants emphasized the moral value of the oath both in terms of content and for themselves. Thanks to the technology that eliminates the concept of time and place during the pandemic period, students have received the theoretical training they should have received.

According to a study conducted on university students in Turkey, distance education attitude was not affected by gender, age, or demographic characteristics. However, university students experienced more anxiety in obtaining a profession than in regular times due to pandemic restrictions. Women were particularly affected by this process. Students were most affected by the epidemic and the uncertainty in the education process (Öz Ceviz, 2020).

Studies suggest that medical students are more affected than other students. Unfortunately, the inability to participate in laboratory studies and receive adequate training is an important concern for health care provider candidates. More than half of the physician candidates stated that their social relations deteriorated, and it is necessary to take precautions for their mental well-being (Seetan, 2021).

Morality is an abstract concept. Deontology universally advises physicians. The unpretentious promises of the students that they will obey the rules are an example of a concrete transition to practical life(Helmich& Carvalho-Filho, 2018; Prabhumoye, 2021). Participants stated that they felt bad because of the pandemic, but the swearing-in ceremony made them happy. The students welcomed the repeated action of the swearing-in ceremony for the students. It can be argued that the practice, carried out to the extent possible, makes the students feel good spiritually.

As a result, the oath of Mersin University Faculty of Medicine contains statements that are dynamically realized following the developing education vision and reflect contemporary medical ethics. In addition, the oath ceremonies held during the pandemic restrictions contributed to the students' understanding of medicine as an ethical profession. The guiding and binding effect of the oaths took place in the students' statements, and it is thought that it will be reflected in their clinical practices.

Oaths have been an essential ritual for physicians for centuries; even if there are pandemic restrictions, performing these ceremonies will help physician candidates to make sense of their existence in the profession. The pandemic has negatively affected the professional and private lives of all healthcare professionals, and it is important to take actions similar to our work, which makes you smile and refers to the positive features of the profession.

Acknowledgments

We would like to thank Dr. Seyhan Oktar, Dr. Bilal Hamad, and Dr. Kübra Zinnur for their assistance in our data collection. This study is dedicated to all healthcare workers who died during the Corona pandemic. Bless their souls.

Hipocratic Oath Ceremony 2020 (Mersin University) Available at:

Mersin Üniversitesi Tıp Fakültesi 2020 mezunları Hippokrat yemini - YouTube

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Chapter 17

PIWIs AND THEIR ROLES IN HUMAN CANCERS

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Introduction

A novel gene whose mutations eliminate division of germline stem cell in the Drosophila ovary was discovered by Lin et al. in 1997. This gene has been named as P-element-induced wimpy testis (PIWI) due to P-element insertion mutations causing a severe defect in spermatogenesis and male infertility (Lin et al., 1997). In many organisms, PIWI proteins, member of the argonaute protein family, have crucial from the initial stage of germline development to the final stage of gametogenesis (Thomson et al., 2009).

PIWI proteins plays indispensable roles in piRNA biogenesis (as detailed in chapter 2), as well as in the piRNA-induced silencing complex (piRISC) and regulation of gene expression by chromatin rearrangement. PIWI proteins have critical roles in the epigenetic and genetic regulation of gene expression (Ghildiyal et al., 2009). piRNAs are required to complex with PIWI proteins for gene expression silencing, histone modification, transposon silencing, and DNA methylation (Akkouche et al., 2013). Therefore, PIWI are crucial in small RNA-mediated gene silencing through piRNAs. PIWI proteins contains the N-terminal PAZ domain that binds to the 3' end of the guide RNA, the MID domain that binds to the 5' end of the guide RNA, and the C-terminal PIWI domain with RNAse H activity (Figure 1). PIWI- like protein 1 (PIWIL1/ HIWI), PIWIL2 (HILI), PIWIL3 (HIWI3) and PIWIL4 (HIWI2) are the four PIWI proteins found in humans (Sasaki et al., 2003). Mouse PIWI proteins are defined as MIWI, MIWI2 and MILI. These are very crucial for spermatogenesis (Balmeh et al., 2021). Three PIWI proteins are determined in Drosphila including Argonaute 3 (AGO3), Aubergine (Aub) and PIWI. Because PIWIs are expressed in both germ and somatic cells, they have critical roles in piRNA pathways in both cells (Guo et al., 2013; Pippadpally&Venkatesh et al., 2020). DNA methylation is a crucial machinery in PIWI-mediated epigenetic silencing. Therefore, it has been thought that piRNAs also induce methylation at non-transposon loci (Peng & Lin 2013).

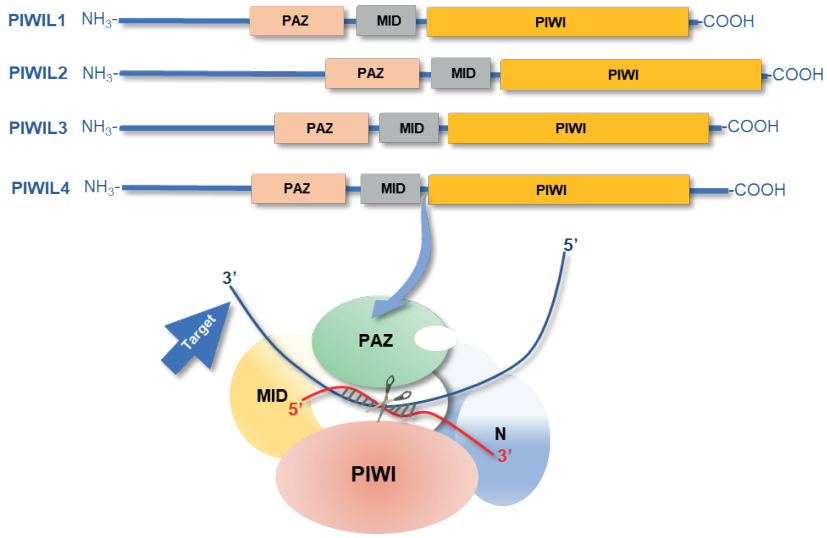

Figure 1. *Structure of PIWIs (Adapted from Angelova 2018; Bamezai et al., 2012).*

PIWIL1 has a crucial role in spermatogenesis. PIWIL2 plays an essential function in stem cell self-renewal of germline, silencing of fetal gonocytes and spermatogenesis. In addition, tudor domain-containing protein 1 (TDRD1) has a critical function in piRNA biogenesis by forming a direct complex with PIWIL1 and PIWIL2 (Ferreira et al., 2014). PIWI proteins have also been thought to be important in self-renewing diseases such as cancer due to their stemness abilities (Siddiqi&Matushansky 2012a).

Abnormal expression of PIWIs in various cancers

PIWIs are generally expressed in germ cells, stem cells and cancer cells in adults (Siddiqi&Matushansky 2012a). Although it was stated that the PIWIL1 is not expressed in somatic tissues, the results of the research have been illustrated that it is expressed in germ cells and stem cells found in tumor tissues (Siddiqi et al., 2012b). There are studies showing PIWIL2 expression in many cancers including renal cell carcinoma (Iliev et al., 2016), colon cancer (Li et al., 2012) and non-small cell lung cancer (Qu et al., 2015) besides its functions in germ cell development. It has been shown that PIWIL2 is highly expressed in tumors and suppressed apoptosis by activating the Stat3/Bcl-XL pathway (Lee et al., 2006). PIWIL2 plays a pivotal role in piRNA biogenesis and has aberrant expression in malignancies (Erdogdu et al., 2018). PIWIL4 is expressed in most human tissues and its expression is known to be high in cancer tissues (Han et al., 2017). Studies on the roles of PIWI proteins in cancer are being carried out and it has been shown to be important in processes including invasion (Li

et al., 2012), apoptosis (Lee et al., 2010), cell viability (Liu et al., 2006) and metastasis (Wang et al., 2012). Moreover, these proteins have been found to be important markers for diagnosis and/or prognosis (Sun et al., 2011; Wang et al., 2012). Previous research indicated that the expression of PIWI proteins is abnormal in cancer (Balmeh et al., 2021). Therefore, PIWIs can be a more specific treatment option targeting cancer cells. The roles of PIWIs (Figure 2-4) in various cancers are given in detail below.

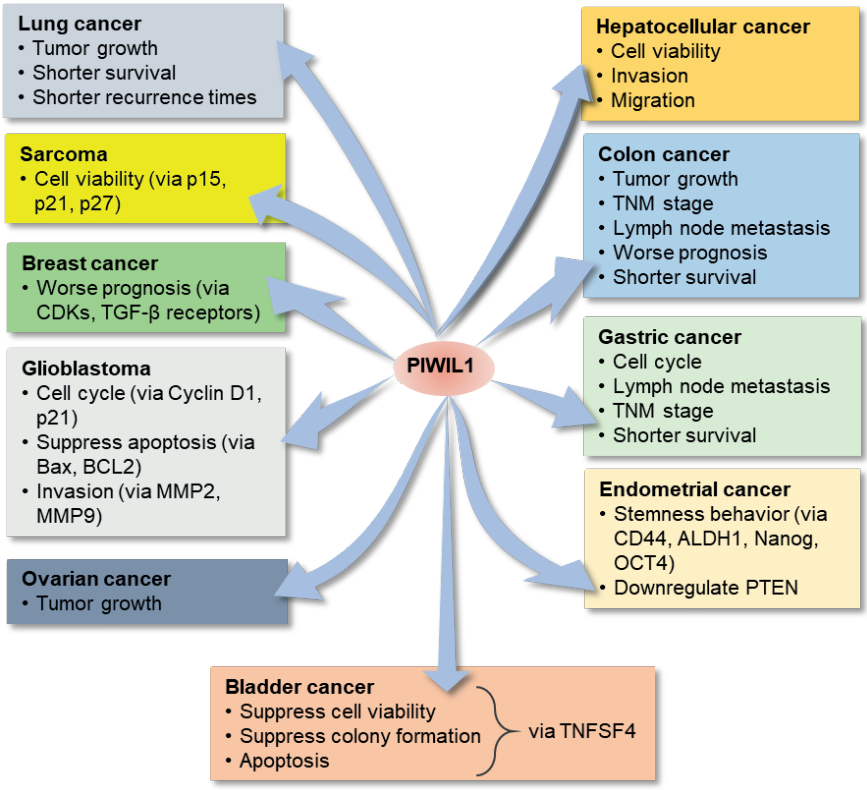

Figure 2. *Mechanism of PIWIL1 in common cancers*

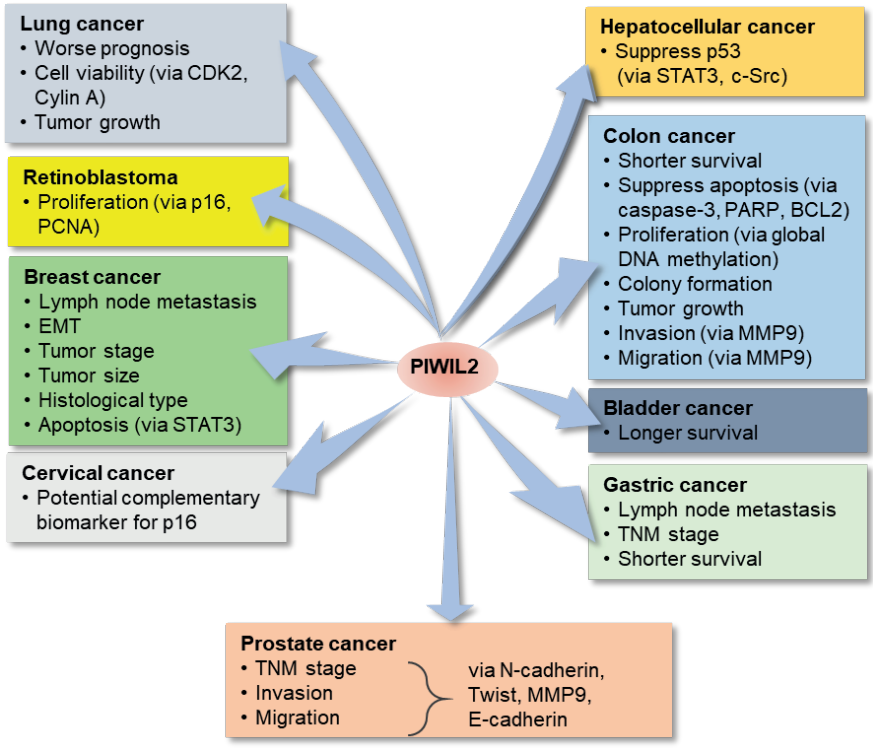

Figure 3. *Mechanism of PIWIL2 in common cancers*

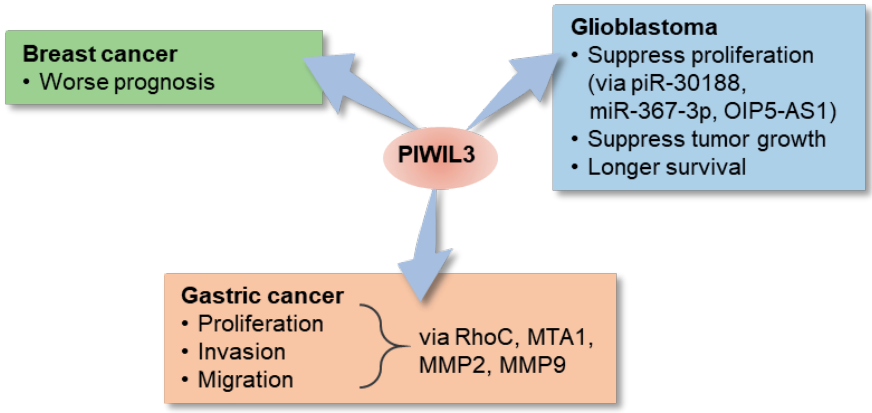

Figure 4. *Mechanism of PIWIL3 in common cancers*

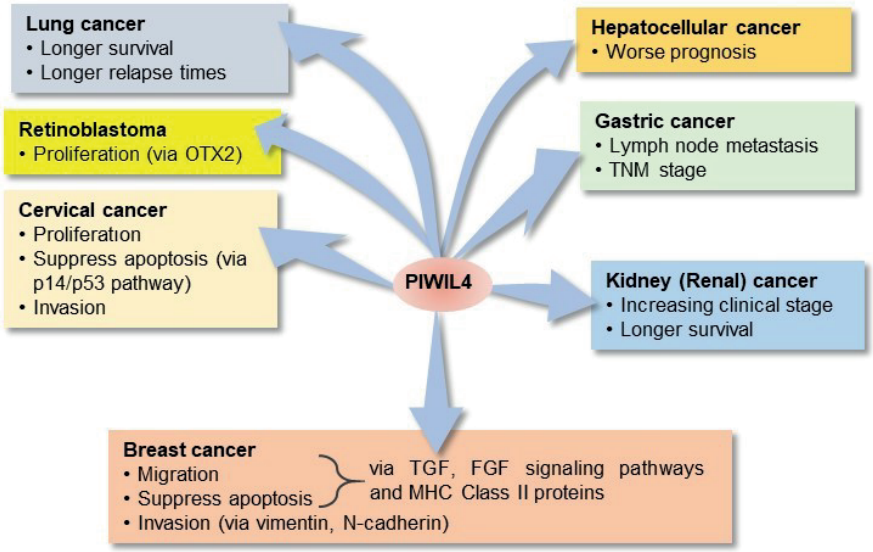

Figure 5. *Mechanism of PIWIL4 in common cancers*

Lung cancer

PIWIL1 expression is upregulated in SSCloAldebr cells defined as lung cancer stem cells compared to lung adenocarcinoma cells SPC-A1. Suppression of PIWIL1 has decreased sphere and colony formation in SSCloAldebr cells. PIWIL1 knockdown in SSCloAldebr cells has suppressed tumor growth in xenograft mice. Therefore, PIWIL1 is thought to play an important role in the stemness properties of cancer stem cells in lung cancer and may be an important target in lung cancer therapy (Liang et al., 2013). Results of a study showed that some of the lung cancer patients had expression of PIWIL1 while others did not. Moreover, it was concluded that patients with PIWIL1 expression had shorter survival and recurrence times. The expression of PIWIL2 and PIWIL4 was found to be lower in lung cancer tissues compared to non-cancer tissues. It has been reported that patients with low PIWIL4 expression have shorter survival and relapse times (Navarro et al., 2015). PIWIL2 expression is higher in non-small cell lung cancer (NSCLC) tissues than in surrounding non-cancerous tissues. High expression of PIWIL2 has been associated with a poorer prognosis in NSCLC patients. Overexpression of PIWIL2 has increased proliferation in NSCLC cells by activating of CDK2 and Cyclin A, while knockdown of PIWIL2 has decreased cell viability by causing cell cycle arrest in the G2/M stage. Moreover, in the xenograft model with NSCLC, PIWIL2 induced tumor growth (Qu et al., 2015).

Prostate cancer

PIWIL2 gene expression has been found to be associated with TNM and gleason score in prostate cancer tissues. PIWIL2 knockdown has caused suppression of invasion and migration by decreasing N-cadherin, Twist, vimentin and MMP9 expressions and increasing E-cadherin expression in prostate cancer cells. These results suggest that PIWIL2 could be a potential target in prostate cancer treatment (Yang et al., 2015a).

Breast cancer

PIWIL1 expression is upregulated in both breast cancer tissues and cells. Elevated expression of PIWIL1 was associated with lymph node metastasis, tumor size and histologic grade. PIWIL1 caused increased cell growth in both breast cancer tissues and breast cancer cells (Wang et al., 2014a). The results of another study showed that PIWIL1 expression was elevated in breast cancer tissues. In addition, high expression of PIWIL1 has been associated with worse prognosis through cyclin dependent kinases and TGF-β receptors. On the other hand, suppression of PIWIL1 expression has caused arrest in the G2/M phase of breast cancer cells. These results suggest that PIWIL1 may be a prognostic factor in breast cancer (Cao et al., 2016).

PIWIL2 protein level is higher in breast cancer tissues compared to non-cancerous tissues. PIWIL2 expression is positively correlated with and lymph node metastasis in postoperative cases. PIWIL2 may play a critical function as a positive epithelial mesenchymal transition (EMT) regulator in breast cancer stem cells through methylation of latexin (a tumor suppressor protein) by binding to piR-932. PIWIL2 has been associated with tumor stage, tumor size, histological type, lymph node metastasis and age in breast cancer (Zhang et al., 2013). Expression of PIWIL2 in breast cancer tissues was highest in 90% of invasive carcinomas and 81% of *in situ* carcinomas. Knockdown of PIWIL2 expression induced apoptosis by suppressing STAT3 expression in breast cancer cells (Lee et al., 2010).

PIWIL2 and PIWIL4 expression levels are significantly lower breast cancer tissues. Expressions of PIWIL1 and PIWIL3 have been upregulated in breast cancer tissues but not statistically significant. PIWIL3 and PIWIL4 have been associated with prognosis in breast cancer compared to breast tissues obtained from mammoplasty (Krishnan et al., 2016). PIWIL4 expression has been reported to be upregulated in both breast cancer tissues and cells. Silencing of PIWIL4 resulted in suppression of migration and increased apoptosis in breast cancer cells. In addition, it has been shown that PIWIL4 partially caused these effects by regulating TGF, FGF signaling pathways and MHC class II proteins. For these reasons, PI-

WIL4 seems to be a therapeutic target in breast cancer (Wang et al., 2016). Expression of PIWIL4 in breast cancer was increased by 17β-estradiol treatment. Knockdown of PIWIL4 expression has suppressed invasion and migration by decreasing mesenchymal markers including vimentin and N-cadherin expressionsin breast cancer cells (Heng et al., 2018).

Colon cancer

PIWIL1 expression is upregulated in colorectal cancer tissues compared to surrounding non-cancerous tissue. High PIWIL1 expression has positively been correlated with TNM staging and lymph node metastasis. It has also been seen that patients with high PIWIL1 expression have a worse prognosis and lower survival. In the light of these data, PIWIL1 can be a prognostic marker in colorectal cancer patients (Sun et al., 2017). Another *in vitro* study has shown that PIWIL1 was upregulated in colon cancer tissues. In addition, PIWIL2 overexpression has been shown to increase colon cancer cell proliferation, which has been associated with an increase in global DNA methylation (Yang et al., 2015b). Upregulated PIWIL1 expression and downregulated PIWIL2 expression in mRNA level has been shown in colon cancer tissue compared to adjantment non-cancer tissue. PIWIL1 expression has been associated with OCT4 expression in cancer tissues. PIWIL2 expression has also been shown to be positively correlated with SOX2 expression in cancer tissues. These findings indicate that PIWIL1 and PIWIL2 are important in the regulation of some cancer stem cell markers (Litwin et al., 2015).

PIWIL2 expression is upregulated in colon cancer tissues compared to normal colon mucosa. PIWIL2 level is positively correlated with poorer five-year survival and poorer clinic and pathologic situation. PIWIL2 knockdown has induced apoptosis and suppressed proliferation and colony formation in colon cancer cells. Silencing PIWIL2 has reduced tumor growth in vivo. Moreover, PIWIL2 has modulated invasion, migration by regulating matrix metallopeptidase 9 (MMP9) at the transcriptional level and apoptosis via caspase-3, PARP and BCL2 in colon cancer (Li et al., 2012). PIWIL2 expression has been reported to be higher in approximately 73% of patients with colorectal cancer. Higher expression of PIWIL2 has been related deep invasion, differentiation and perineural invasion. PIWIL2 expression has been shown to be negatively correlated with overall survival of colon cancer patients (Oh et al., 2012).

Hepatocellular cancer

PIWIL1 expression is high in both hepatocellular carcinoma (HCC) cells and tissues. Knockdown of PIWIL1 has resulted in suppression of viability, invasion and migration of HCC cells. These findings suggest that PIWIL1 may have an oncogenic role in hepatocellular carcinoma and

thereby a potential therapeutic target (Xie et al., 2015). PIWIL2 has been shown to suppress a tumor suppressor p53gene in HCC cells. Immunoprecipitation experiments suggested that PIWIL2 can directly interact with STAT3. It has been concluded that PIWIL2 and STAT3 can also form complexes with c-Src. Thanks to this complex, STAT3 is phosphorylated by c-Src and translocated to the nucleus. Then, STAT3 binds to promoter region of P53 to suppress the transcription. This indicates that PIWIL2 may have an oncogenic role in human cancers (Lu et al., 2012). PIWIL2 expression in HCC tissue samples has been reported to be higher in the nucleus than in the cytoplasm. PIWIL4 expression was found to be lower in the nucleus than in the cytoplasm in the same tissues. Four co-expression patterns of PIWIL2/PIWIL4 have been identified including (i) co-expression in nucleus, (ii) co-expression in cytoplasm, (iii) co-expression in nucleus and cytoplasm and (iv) non-coexpression. It has been shown that patients with hepatocellular carcinoma in which PIWIL2/PIWIL4 is co-expressed in the nucleus have a worse prognosis compared to patients in whom PIWIL2/PIWIL4 is not co-expressed. Therefore, it has been concluded that co-expression of PIWIL2 and PIWIL4 in the nucleus causes worse prognosis in HCC patients (Zeng et al., 2017).

Only PIWIL2 and PIWIL4 genes are expressed in normal liver cells. No significant expression of PIWIL1 and PIWIL3 has been found in either HCC or the adjacent non-cancerous liver tissues. However, PIWIL2 and PIWIL4 expressions have been seen to be high in hepatocellular carcinoma (Law et al., 2013).

Ovarian cancer

PIWIL1 expression is upregulated in ovarian cancer tissues compared to normal ovarian and benign ovarian tissues. PIWIL3 expression is downregulated. Interestingly, PIWIL2 expression has been reported to be high in stromal cells of ovarian cancer. Overexpression of PIWIL1 has been shown to suppress invasion in ovarian cancer cells (Lim et al., 2014). Expressions of PIWIL1, PIWIL2 and PIWIL4 are higher than serous ovarian cancer tissues compared to normal ovarian tissues. In the endometrioid ovarian cancer tissues, PIWIL2 and PIWIL4 expressions were downregulated; however, PIWIL1 level was higher than non-cancerous tissues (Singh et al., 2018).

Bladder cancer

PIWIL1 expression has been found to be downregulated in bladder cancer tissues compared to adjacent normal bladder tissues. It has also been shown that PIWIL1-piR-ABC complex can regulate cell viability, colony formation and apoptosis via tumor necrosis factor (TNF) superfamily member 4 (TNFSF4) in bladder cancer (Chu et al., 2015).

Lower expression of PIWIL2in cytoplasm and negative expression in nucleus has been associated with worse disease-specific survival and shorter progression-free survival in bladder cancer patients having chemotherapy treatment. For this reason, it has been thought that PIWIL2 may be a prognostic marker in bladder cancer (Taubert et al., 2015).

Endometrial cancer

PIWIL1 expression was found to be high in endometrial cancer tissue and positively correlated with malignant potential. Moreover, PIWIL1 triggered stemness behavior of endometrial cancer cells via CD44, ALDH1 cancer stem cell markers and Nanog and Oct4 stem cell markers (Chen et al., 2015a). Other studies also shown that PIWIL1 expression is high in endometrial cancer. However, there was no relationship between PIWIL1 expression and endometrial cancer clinicopathological features (Liu et al., 2010). PIWIL1 has been shown to suppress PTEN expression in endometrial cancer cells by causing PTEN methylation via DNA methyltransferase 1 (DNMT1). Knockdown of DNMT1 expression resulted in increased PTEN gene expression. In other words, PIWIL1 suppressed the expression of the tumor suppressor gene PTEN by causing hypermethylation of the PTEN gene promoter in endometrial cancer. Therefore, a new regulatory mechanism of PIWIL1 in endometrial cancer was revealed (Chen et al., 2015b).

Glioblastoma

Suppression of PIWIL1 expression has caused cell cycle arrest and induction of apoptosis by changing cyclin D1, p21, Bax and BCL2 expressions in glioma cells. In addition, PIWIL1 knockdown has suppressed invasion and migration by causing a decrease in MMP2 and MMP9 expression in glioma cells. Knockdown of PIWIL1 has inhibited glioma growth in an in vivo mouse xenograft model. These findings suggest that PIWIL1 plays an oncogenic role in glioma (Wang et al., 2014b). PIWIL1 expression has been shown to be high in glioblastoma tissues and positively correlated with tumor grade. In glioma patients, those with higher expression of PIWIL1 have been reported to have poorer overall survival compared with patients having lower PIWIL1 expression. These findings suggest that PIWIL1 may be a diagnostic and prognostic marker in glioma progression and malignant gliomas (Sun et al., 2011). It has been reported that PIWIL3 expression was lower in glioma tissues and cells. Overexpression of PIWIL3 has inhibited glioma cell proliferation. In addition, it was concluded that this inhibition was mediated by piRNA (piR-30188), miRNA (miR-367-3p) and lncRNA (OIP5-AS1) containing target binding sites. In addition, *in vivo* overexpression of PIWIL3 has been associated with inhibition of tumor growth and longer survival (Liu et al., 2018).

Gastric cancer

The expression rate of PIWIL1 has been investigated in normal gastric (10%), atrophic gastritis (36%), intestinal metaplasia (36%) and gastric cancer (76%) tissues and was found to be positively correlated with cancer development. In addition, the PIWIL1 has similar expression pattern with Ki67, which is used as a proliferation marker, in gastric cancer tissues. PIWIL1 knockdown has caused suppressing of cell proliferation by inducing cell cycle arrest in G2/M phase in gastric cancer cells (Liu et al., 2006).

Expressions of PIWIL1-4 have been upregulated in gastric cancer tissues compared to adjacent normal gastric tissue. Expressions of PIWIs have been shown to be positively correlated with lymph node metastasis, T, and clinical TNM stage. Moreover, high expressions of PIWIL1 and PIWIL2 have been found to be associated with poor overall survival. Therefore, PIWIL1 has been shown to be an independent prognostic factor in gastric cancer patients (Wang et al., 2012).

PIWIL3 expression has been shown to be elevated in gastric cancer tissues. Knockdown of PIWIL3 has suppressed proliferation by causing cell cycle arrest in the GO/G1 phase, invasion and migration by reducing RhoC, MTA1, MMP2 and MMP9 expressions in gastric cancer cells (Jiang et al., 2017).

Kidney (renal) cancer

PIWIL1 expression has been shown to be downregulated in renal cell carcinoma tissues. Although no significant changes were observed in PIWIL2 and PIWIL4 expressions, PIWIL1, PIWIL2 and PIWIL4 expression levels were negatively correlated with increasing clinical stage. Moreover, low expression of these PIWIL genes has been associated with poorer survival in renal cell carcinoma patients. This findings suggested that other PIWIL genes, except PIWIL3, may be prognostic biomarkers in renal cell carcinoma (Iliev et al., 2016). Expressions of PIWIL1, 2 ve 4 in transcriptional level has associated with each other in renal cell carcinoma tissues and non-cancerous tissues. PIWIL4 expression has been observed to be upregulated in renal cell cancer tissues. It has been reported that PIWIL1 expression is higher in young patients with renal cell cancer (Al-Janabi et al., 2014).

Testicular cancer

Specific expression of PIWIL1 has been demonstrated at important stages of spermatogenesis in germ cells. Moreover, PIWIL1 overexpression has been associated with seminoma germline tumors (Qiao et al., 2002). Ferreira et al. (2014) reported that PIWIL1, PIWIL2 and PIWIL4

were epigenetically suppressed by hypermethylation of promoter region CpG islands in both testicular germ cell tumor cells and non-seminoma and primary seminoma testicular tumors. Expressions of PIWIs have been interestingly suppressed in testicular germ cell tumors compared to normal testis. An another study supported this findings by showing the presence of PIWI/piRNA biogenesis was absent in germ cell neoplasia in situ and testicular germ cell tumors. In addition, the presence of piRNA biogenesis in germ cells in healthy adult testes has been reported (Gainetdinov et al., 2018).

Sarcoma

Elevated PIWIL1 expression suppressed the differentiation of sarcoma precursors *in vitro* and causes sarcoma formation in vivo. Downregulation of PIWIL1 have suppressed proliferation and trigged differentiation in human sarcomas. It has been concluded that PIWIL1 has oncogenic properties in sarcoma. It has been shown that cyclin-dependent kinase inhibitors (CDKI) including p15, p21 and p27 were silenced through PIWIL1-mediated DNA methylation (Siddiqi et al., 2012b).

Cervical cancer

PIWIL2 has been shown to be expressed in different stages of cervical cancer. Different expression of PIWIL2 was observed in premalignant and malignant lesions. However, p16 expression, which is important indicator in human papillomavirus infections, was also detected in approximately 84% of these samples. However, it was observed that p16 and PIWIL2 were coexpressed and their expressions were often absent. Therefore, it is thought that PIWIL2 may be a complementary biomarker with P16 in cervical cancer (He et al., 2010).

Expression of PIWIL4 is higher in cervical cancer tissues compared to non-cancerous tissues. PIWIL4 has been shown to increase proliferation and suppress apoptosis by inhibiting the p14/p53 pathway. Moreover, PIWIL4 has increased the invasion ability of cervical cancer cells. In line with these findings, it is suggested that PIWIL4 may have an oncogenic role in cervical cancer and seems to be a therapeutic target in the treatment of cervical cancer (Su et al., 2012).

Melanoma

PIWIL3 expression has been found to be higher in primary melanoma compared to metastatic melanoma. PIWIL3 expression was positively correlated with tumor growth in primary melanoma (Gambichler et al., 2017).

Retinoblastoma

Expression of PIWIL4 has been shown to be high in retinoblastoma cells. PIWIL4 knockdown caused no change of levels of stem cell markers including Oct-3/4, Sox-2 and Nanog. However, it reduced the expression of OTX2, which is an important gene in eye development. In addition, suppression of PIWIL2 caused arrest in the G2/M phase and reduced proliferation via proliferating cell nuclear antigen (PCNA) and tumor suppressor gene P16 in retinoblastoma cells (Sivagurunathan et al., 2017).

Conclusion

PIWI proteins have crucial functions in piRNA-induced silencing and piRNA biogenesis. Now, it is known that the expression of the four PIWI proteins is abnormal in many human cancers. In this book chapter, the molecular mechanisms of PIWI proteins in many cancers are summarized. There is need for further studies to understand these mechanisms, which have capacity of PIWI proteins as prognostic and/or diagnostic markers in many cancers.

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Chapter 18

EFFECT OF THE VARIABLES OF SEX AND MARITAL STATUS AMONG NURSES IN WORK- FAMILY CONFLICTS: A META-ANALYSIS STUDY*

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* This study was produced from the doctoral thesis of the first author. Doctoral thesis name is "Effect of the Variables of Sex and Marital Status among Nurses in Work-Family Conflicts: A Meta Analysis Study".

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INTRODUCTION

Family that is the smallest unit of a society from existence of human being to present day, is a basic and universal institution. Business, on the other hand, is a concept allowing individuals realize their roles related to family and defining continuance of their lives and positions in a society. Business and family areas take place in the center of human lives and take amongst the areas demanding individual's energy and time at the highest level. An individual is held responsible from roles given in work life, in addition to roles except work life when that individual begins working. Therefore, an individual with limited sources and time, faces with difficulties to keep these two areas balanced while trying to realize work and family areas' expectations. When a balance could not be established between work and family areas, work- family conflict is experienced. Netemeyer et.al. (1996) defined work-family conflict as work and family responsibilities intervene or prevent each other. Wayne et.al. (2004) defined work-family conflict as role expectations of work and family lives are not complying. Experienced conflict is named as "Business-Family Conflict (İAÇ) when family tasks are prevented by work responsibilities; as "Family-Business Conflict (AİÇ) when family responsibilities prevent tasks related to work (Kılıç ve Sakallı 2013: 213; Frone, Russel and Cooper 1992:728).

Work-family conflict can arise sourced from individual factors such as gender, marital status, education, age, etc., it can also arise as a result of family sourced factors such as number of children, having individual dependent (elderly, disabled, etc.), relationships with family, child care, individual not participating family related activities, spouses working in married families, having no spouse assistance or less assistance. Also organizational factors such as extreme work load, extreme working hours, irregular working hours, extra work, working in shifts (day-night), administrator attitude, role load in work and family areas, working conditions, double career family structure, can also cause work-family conflict for an individual(Turunç ve Erkuş 2010:418).

Majority of nursing occupation is formed by women. Factors such as difficult working conditions, long working hours, shift works and extreme work load, complicate nurses to perform their work and family responsibilities, cause nurses to have difficulties in balancing their work and family lives. Due to this situation, nurses consider themselves insufficient in performing their responsibilities. Although work-family conflict arises as a result of increasing nurses' anxiety level in addition to make them leave less time for their responsibilities in their family life.As a result of increase in the number of male nurses in recent years, having male nurses experience work-family conflict caused us to ask whether gender has an

effect on work-family conflict.Although there are studies demonstrating that marital status has an effect on work-family conflict in nursing, especially due to the fact that roles and expectations for family area, are too much, there are studies demonstrating that married nurses experience much more work-family conflict than single nurses. This situation causes us to question the effect of being single or married on work-family conflict and which of the groups experience more work-family conflict.

By considering responsibilities shouldered by multiple roles of nurses (woman-spouse-mother, etc.), it is thought that they could oscillate between their work and family responsibilities and it is inevitable for them not to experience work-family conflict in that intensive tempo. Additionally, in the results of studies made on the literature (Gamor et. al., 2014: 1-8; Mcnamara et. al., 2013), importance of flexible working applications has been stressed in decreasing experienced work-family conflict. This this study, by analyzing the studies related to work-family conflict experienced by nurses, taking place in the literature with meta-analysis program, it has been aimed to reach stronger and certain results related to gender and marital status variations affecting work-family conflict in terms of individuals and organizations.In sociodemographic variations, gender and marital status have been taken as basis since variations such as amongst the other variations such as age, education, having child etc., have lack of sufficient data or have fewer data and each variable includes a detailed research.Therefore, our study is a meta-analysis study executed with the purpose of determining whether gender and marital status variations of nurse have an effect on work-family conflict.

The Purpose of the Research

The purpose of this research is to determine effect of gender and marital status experienced by nurses, on work-family conflict.

2. Method

2.1.The Research Method

Meta-analysis method that is one of synthesizing methods for quantitative research results, forms the model of this study. Before collecting data, PICOS method was practiced, research questions and key words were been determined. Created research questions have been given below:

- What is effect size of marital status variable on work-family conflict experienced by nurses?
- What is effect size of gender variable on work-family conflict experienced by nurses?

2.2. Collecting Data

Articles, PhD dissertations and postgraduate dissertations taking place in national and foreign literature, have formed data sourced of this research. Turkish Council of High Education National Thesis Center where dissertations are archived in Turkey and ULAKBİM Health Sciences Data Base where academic dissertations are archived and Web of Science, CINAHL, PUBMED and ProQuest Digital Dissertations data base for foreign publications, have been scanned.

While scanning, keywords of "work-family conflict","nurse", have been used in Turkish and English. While scanning, these keywords have been searched in studies' titles, summaries and keywords. Keywords have also been used in combination in addition to using them one by one. After scanning, the ones suitable with inclusion criteria amongst the ones reached in full form, have been determined and included into meta-analysis. When it has been encountered with multiple studies containing the same data source (thesis or article), thesis or articles that has more information, has been included into the study.

2.3.Data Collecting Tool

"Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA)" model software guide that is used for systematic reviews and meta-analysis, have been used in reporting this study (Moher, Liberati, Tetzlaff and Altman, 2009). In order to determine practicing characteristics of articles and thesis, PRISMA Declaration form containing 27 items, has been used (Table 1). Turkish translation of Data Retrieval Form/Checklist taking place in the guide, was performed by Aşık and Özen (2019), its Turkish version published on official internet site of PRISMA, was used(Aşık ve Özen, 2019; Prisma Checklist, 2019). Response options given while filling the form, are "Appropriate/Yes =1 point", "Not Appropriate/No=0 point", "Partly Appropriate=0,5 point" and "Non-applicable". Besides, before data collection process, the researchers (the researcher and thesis advisor) discussed articles of PRISMA Declaration in terms of comprehensibility. The articles have been evaluated independently, and a consensus has been reached regarding comprehensibility of the items. Consistency between the researcher and the other observer (advisor) in the study, has been made with Cohen's Kappa analysis, its compliance **rate has been found as 0,83 (very good compliance).**

Document	Covered by PRISMA items	PRISMA Response Scores			
		Appropriate / Yes (1 point)	Not Appropriate / No (0 point)	Not applicable	Partly Appropriate (0,5 point)
Title	item 1				
Abstract	item 2				
Introduction	item 3,4				
Methods	item 5,6,7,8,9,10, 11,12,13,14,15,16				
Results	item 17,18,19,20, 21,22,23				
Discussion	item 24,25,26				
Financing resources	item 27				

Table 1. *Determination strategy of the studies to be included in the Meta-Analysis study*

The study covers publications and dissertations those were made in between 01.01.2009 and 31.12.2019 and those could be reached. Population of the study that has been executed to determine effect of work-family conflict experienced by nurses based on marital status and gender variations, has been formed by 5887 articles and/or dissertations obtained as a result of scanning made through texting "work-family conflict" (Figure 1).

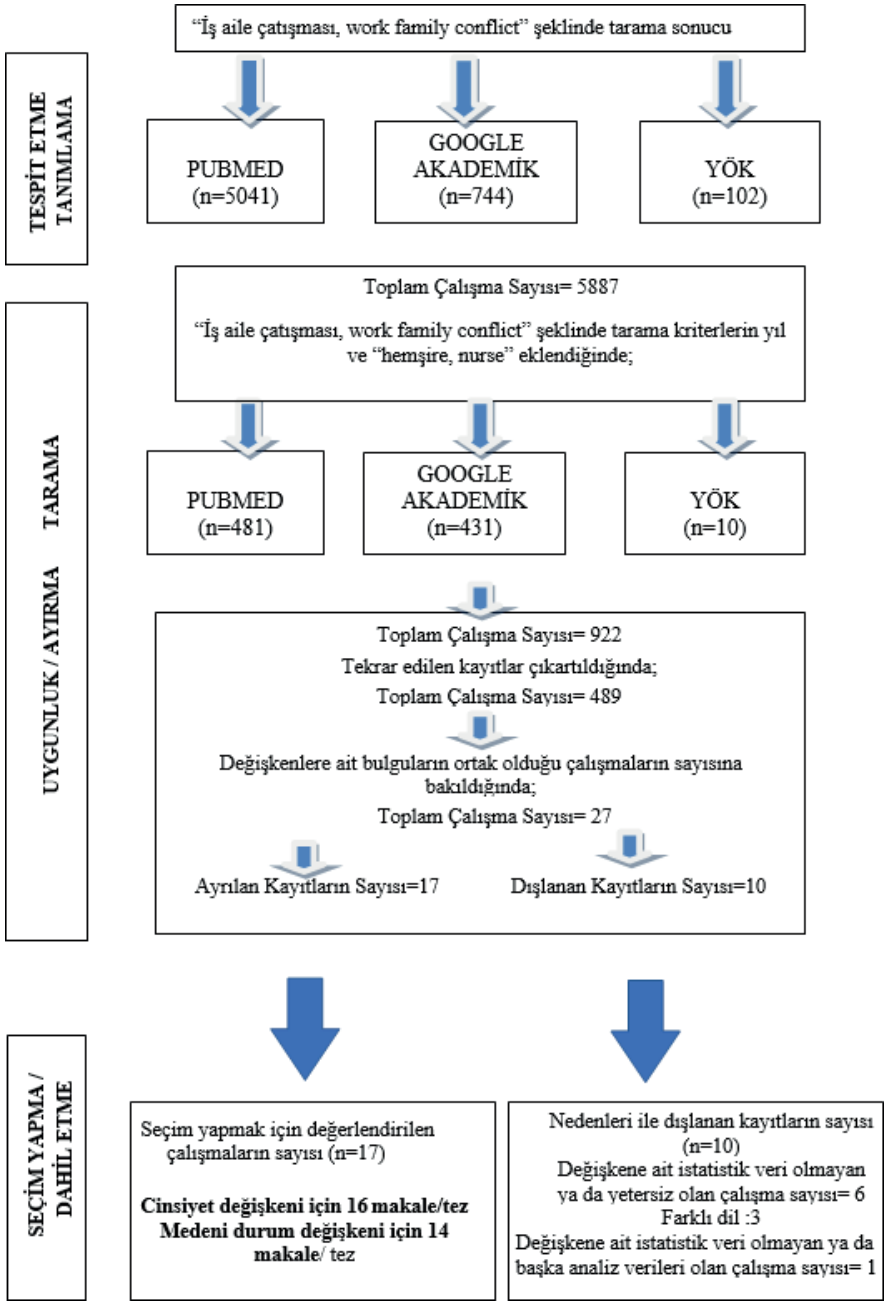


Figure 1. *PRISMA flow chart used in the selection of studies*

When year and nurse words suitable for inclusion criteria were added to scanning criteria, we reached 822 studies and when repeated records were taken out, we reached 489 studies.When studies where findings related to variations

were taken into consideration, we reached 27 studies. Amongst 27 studies found, 10 studies in total were not included into the study; 3 studies that have not statistical data for variable or that are insufficient, 3 studies written in a non-English language and 1 study that has different analysis date for variable, 17 studies formed the study sample (Table 2).

Table 2. *List of studies included in the meta-analysis*

ÇALIŞMA LİSTESİ	ARAŞTIRMACI	KAYNAK
1. Hemşirelerin İş Yükü Algısının İş-Aile Çatışması Üzerine Etkisinin İncelenmesi: Kahramanmaraş Sütçü İmam Üniversitesi Sağlık Uygulama Ve Araştırma Hastanesinde Çalışan Hemşireler Üzerinde Bir Araştırma	Göde, 2019	Kahramanmaraş Sütçü İmam Üniversitesi Sosyal Bilimler Enstitüsü Sağlık Yönetimi Ana Bilim Dalı Yüksek Lisans Tezi
2. Bir Üniversite Hastanesindeki Hemşirelerde İş-Aile Çatışması İle Örgütsel Sessizlik Ve Sosyal Destek Algısı Arasındaki İlişkiler	Polat ve ark., 2018	Psikiyatri Hemşireliği Dergisi, Psychiatric Nurs 2018;9(3):195-204 ; DOI: 10.14744/phd.2018.38278
3. Can Job Control Ameliorate Work-family Conflict and Enhance Job Satisfaction among Chinese Registered Nurses? A MediationModel	Ding and etc., 2015	Electronic Physician (ISSN: 2008-5842) May2018, Volume:10, Issue:5, Pages:6864-6867, http://www.ephysician.ir, DOI:http://dx.doi.org/10.19082/6864
4. The Relationship Between Work–Family Conflict and Job Satisfaction Among Hospital Nurses	Alazzam and etc., 2017	Nursing Forum Volume52,No.4,October-December2017, DOI: 10.1111/nuf.12199
5. The Effects of Supervisors' Support and Mediating Factors on the Nurses' Job Performance Using Structural Equation Modeling A CaseStudy	Ravangard and etc., 2015	The Health Care Manager Volume 34, Number 3, pp. 265–276, DOI: 10.1097/HCM.0000000000000068
6. Work–family conflict and enrichment in nurses: between job demands, perceived organisational support and work–family backlash	Ghislieri and etc.,2017	
Journal of Nursing Management, 2017, 25, 65–75		
7. Juggling family and professional caring: Role demands, work–family conflict and burnout among registered nurses in Ghana	Asiedu and etc.,2018	Nursing Open. 2018;5:611–620, DOI: 10.1002/nop2.178
8. Hemşirelerde İş Aile- Aile İş Çatışmasının Tükenmişlik Ve İşten Ayrılma Niyetine Etkisi	Hyusmenova, 2017	İstanbul Üniversitesi Sağlık Bilimleri Enstitüsü Hemşirelikte Yönetim Ana Bilim Dalı Yüksek Lisans Tezi
9. İş Yükü Fazlalığı Algısının Yaşam Kalitesi Üzerindeki Etkisi: İş- Aile Çatışmasının Aracı Rolü	Korkmazer, 2018	
İnönü Üniversitesi Sosyal Bilimleri Enstitüsü Doktora Tezi		
10. İş-Aile Ve Aile-İş Çatışması İle Bireysel Performans Etkileşiminde Meslekî Bağlılığın Aracı Rolü: Hemşireler Üzerinde Bir Araştırma	Aktaş ve ark., 2015	
Doğuş Üniversitesi Dergisi, 16 (2) 2015, 139-154		
11. The Moderatıng Effect Of Perceıved Organızatıonal Support (Pos) In The Impact Of Workload And Workfamıly Conflıct On Organızatıonal Commıtment A Research In Hospıtal Nurse Staffıng	Dorela, 2017	Hacettepe University Graduate School of Social Sciences Business Administration Department Management and Organizational Behaviour Master's Thesis

12. Factors That Influence Nurses' Work-Family Conflict, Job Satisfaction, and Intention to Leave in a Private Hospital inTurkey	Ekici ve ark.,2017	Hosp Pract Res. 2017 Dec;2(4):102-108 doi 10.15171/hpr.2017.25
13. Aile Sağlığı Merkezlerinde Çalışan Evli Hemşirelerin İş – Aile Çatışma Düzeyini Etkileyen Faktörler	Gürel ve ark., 2017	ACU Sağlık Bilimleri Dergisi, 2017 (3), 150-156
14. Hemşirelerin İş Aile çatışması ve Yaşam tatmini Düzeyleri: DemeografiK Özellikler Açısından Bir Değerlendirme	Yıldırımalp ve ark., 2014	Siyaset, Ekonomi ve Yönetim Araştırmaları Dergisi, 2014, Yıl:2, Cilt:2, Sayı :3
15. Work–Family Conflict And Neck And Back Pain İn Surgical Nurses	Baur and etc., 2016	International Journal of Occupational Safety and Ergonomics, Vol 24 (1), 2018, 35-
40 , https://doi.org/10.1080/10803548.2016.1263414		
16. Work–Family Conflict, Cardiometabolic Risk, and Sleep Duration in Nursing Employees.		
Berkman and etc., 2015	Journal of Occupational Health Psychology. 2015 Oct; 20(4): 420–433., doi: 10.1037/a0039143	
17. Work-to-family conflict as a mediator of the relationship between job satisfaction and turnover intention	Chen and etc., 2015	JAN (J Adv Nurs) Leading Global Nursing Research, 2015 Oct;71(10):2350-63. https://doi.org/10.1111/jan.12706

Inclusion and exclusion criteria used for selection researchers, have been given below (Table 3).

Criterion 1:Published or non-published study resources:Scientific articles those have been published on peer-reviewed journals and postgraduate thesis and doctoral dissertations and that has statistic data, shall be included into the study.

Criterion 2:Compliance of research method in the studies:In order to reach effect dimensions in meta-analysis studies, having empirical studies and studies used Work-Family Conflict Scale, shall be taken into consideration.

Criterion 3:Containing sufficient numeric data:In order to calculate effect dimensions required for meta-analysis study, for nurse groups in in the context of variations taken into account; sampling size, average, standard deviation, F value, t value, X2 value, p value and Pearson Correlations Coefficient (r) value shall be taken into consideration.

Exclusion criteria:Not including a study into meta-analysis, is sourced from having out of limitation of the study or not having statistic data required for meta-analysis (Petitti, 2000:33).Therefore, the studies not complying with inclusion criteria, shall be excluded from the study to be used for meta-analysis.

Research security:A coding protocol has been formed for each study included into meta-analysis. In ensuring security of amongst encoders processing studies in coding protocol, Cohen's Kappa statistic was used and security was found as 0,83. This results demonstrated almost a perfect compliance amongst encoders.

Validity of the study:Scanning all studies complying with meta-analysis inclusion criteria and including them into the study are indicators of validity of the study.

Table 3. *Inclusion and exclusion criteria for studies*

Inclusion criteria	Exclusion criteria
Master's and doctoral theses and scientific articles published in peer-reviewed journals	Abstract, proceedings book,publications of the association magazine, editorial comment, interview, letter, news, report, papers
Inclusion of the studies on nurses, use of the "Work Family Conflict Scale"	Doctor, ATT, technician, paramedic, secretary etc. with healthcare workers, not using the ""Work Family Conflict Scale"
Studies published in Turkish-English language	Studies published in a language other than Turkish-English language
Studies carried out between January 1,2009 and December 31, 2019	Studies done before January 1,2009 and studies done after December 31, 2019
Contains sufficient numerical data	Studies without analysis data
Sample size	
Pearson correlation coefficient (r)	
Odds rate	
t-value	
F value	
p- value	
% value	

2.4. Quality of the studies

Quality of the studies indicated in item 12 taking place in PRISMA model, has been demonstrated on Chart 4.Joanna Briggs Institution Critical Assessment Checklist, serves to summarize total points without making separation between low and high quality studies.A point is given to each study.When the lowest point was taken as 0 and the highest as 9 in 17 studies taken into account in the study, it is seen that the studies have been regarding as 8 points (Table 4).

Table 4.*Study quality according to the Joanna Briggs Institute Critical Evaluation Checklist*

	Study	1	2	3	4	5	6	7	8	9	Total
1	Göde, 2019	Y	Y	Y	N	Y	Y	Y	Y	Y	8
2	Polat ve ark., 2018	Y	Y	Y	N	Y	Y	Y	Y	Y	8
3	Ding and etc., 2015	Y	Y	Y	N	Y	Y	Y	Y	Y	8
4	Alazzam and etc., 2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
5	Ravangard and etc., 2015	Y	Y	Y	N	Y	Y	Y	Y	Y	8
6	Ghislieri and etc.,2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
7	Asiedu and etc.,2018	Y	Y	Y	N	Y	Y	Y	Y	Y	8
8	Hyusmenova, 2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
9	Korkmazer, 2018	Y	Y	Y	N	Y	Y	Y	Y	Y	8
10	Aktaş ve ark., 2015	Y	Y	Y	N	Y	Y	Y	Y	Y	8
11	Ekici ve ark.,2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
12	Dorela, 2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
13	Gürel ve ark., 2017	Y	Y	Y	N	Y	Y	Y	Y	Y	8
	Study	1	2	3	4	5	6	7	8	9	Total
14	Yıldırımalp ve ark.,2014	Y	Y	Y	N	Y	Y	Y	Y	Y	8
15	Baur and etc., 2016	Y	Y	Y	N	Y	Y	Y	Y	Y	8
16	Berkman and etc. 2015	Y	Y	Y	N	Y	Y	Y	Y	Y	8
17	Chen and etc., 2015	Y	Y	Y	N	Y	Y	Y	Y	Y	8

Abbreviations: N= No, Y= Yes, U= Unclear, NA=Not Applicaple.

The answer was “no” for item 4 taking place in Joanna Briggs Institution Critical Assessment Checklist. The reason of answering with “no”, was not to place gender and marital status in the titles of the study included into meta-analysis.

2.5. Collecting Data

In this study, statistic Package Program CMA Ver.2.2.064 [Comprehensive Meta-Analysis] was used for Meta-Analysis.There are two important concepts discussed in meta-analytic studies. These are random and fixed effect models (Field, 2005). Accordingly, random effect model has been used in this meta-analysis study.“Fisher’s Z” was used in calculation of effect size and calculations were made by using Comprehensive Meta-Analysis. Interpretation of effect size values were made according to Cohen,Manion and Marrison’s (2007) classification. Accordingly, interpretation was made as follows:

Very week effect between 0,00--0,10

Weak effect between 0,10-0,30

Medium effect between 0,30--0,50

Strong effect between 0,50--0,80

Very strong effect on 0,80 and above.

2.6.Ethical Considerations

The research's protocol has been recorded on "PROSPERO" data base ensuring recording meta-analysis studies and systematic reviews(ID: 198331). There is not ethics comittee approval fort he meta-analysis study. Studies included in the meta-analysis were cited.

3.Findings

17 studies have been include into the study in total. Data related to publication year, publication type, institution that performed these studies and scale of work-family conflict used in these studies, has been given in below charts. It is seen that the studies analyzed relationship between work-family conflict included into meta-analysis and variations (n=17), were began in 2014 and made lastly in 2017. It is seen that the studies executed in 2017, form 35,3% (n=6) of the studied included into meta-analysis. It has been revealed that 23,5% (n=4) of the studies included into meta-analysis, were made in 2015 and 23,5% (n=4) were made in 2018.According to the chart, 66,7% (n=13) of the studies were articles and 33,3% (n=4) were dissertations.Types of dissertations include postgraduate thesis and doctoral dissertations. In the studies included into meta-analysis, 35,4% (n=6) were made in state hospitals, 29,5% (n=5) only in university hospitals, 11,7% (n=2) in private hospitals, 11,7% (n=2) in state and private hospitals and 11,7% (n=2) in state, private and university hospitals. According to the chart, 52,98 (n=9) of the studies included into meta-analysis, were made in Turkey and 11,7% (n=2) in China on nurses. In 76,5 (n=13) of the studies included into meta-analysis, work-family conflict scale developed by Netemeyer, Boles and McMurrian, were used (Table 5).

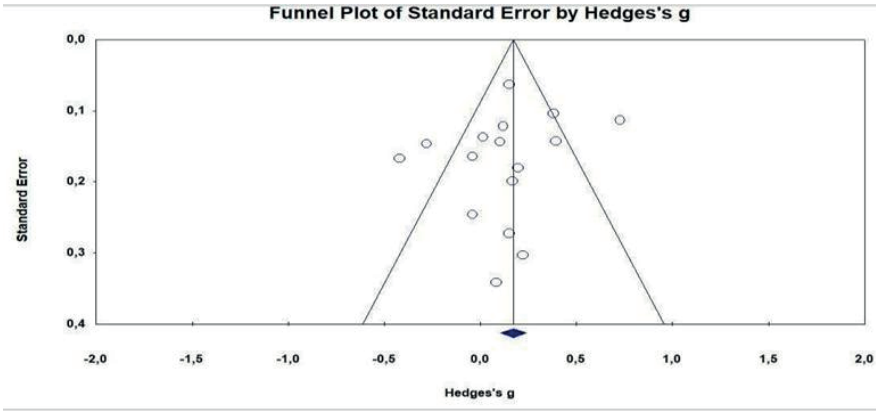
Table 5. *Distribution of studies by year, institution, country, type of publication and used scales*

		Frequency	%
Years of studies	2014	1	5,9
	2015	4	23,5
	2016	1	5,9
	2017	6	35,3
	2018	4	23,5
	2019	1	5,9
Post type	Article	13	66,7
	Thesis	4	33,3
Institution where the studies were carried out	Public	6	35,4
	University Private	2	11,7
	Public and Private	5	29,5
		2	11,7
	Public, University and Private	2	11,7
Country of study	Turkey	9	52,9
	Chinese	2	11,7
	Italy	1	5,9
	Iranian	1	5,9
	Jordan	1	5,9
	Switzerland	1	5,9
	Taiwan	1	5,9
	America	1	5,9
Work Family Conflict Scale	Netemeyer, Boles ve McMurrian Work Family Conflict Scale	13	76,5
	Carlson, Kacmar and Williams Work Family Conflict Scale	3	17,6
	Wayne ve ark Work Family Conflict Scale	1	5,9
	TOTAL	17	100

3.1 Publication Bias

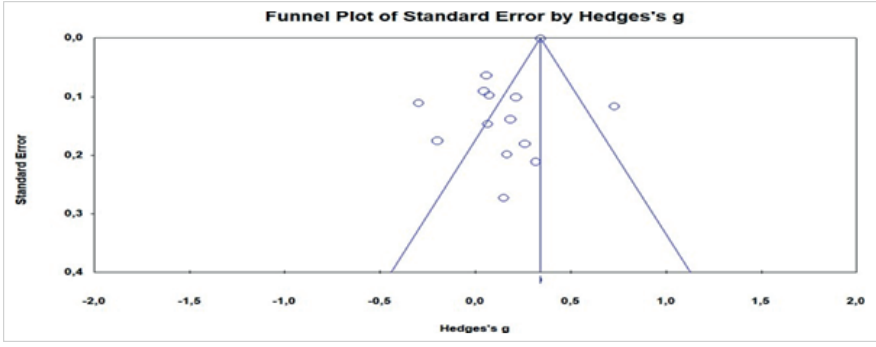
Publication bias of the studies included into meta-analysis for gender and marital status variable, were taken into account separately. In the studies included into meta-analysis for two variations, it has been concluded that there was no publication bias. The most important one amongst them is the diagram named Funnel Plot. Figure 2 has been given for Funnel diagram gender variable and Figure 3 has been given for marital status variable.

Figure 2. *Funnel Plot of Publication Bias by Gender*



It is expected that Tau coefficient in Begg and Mazumdar Rank Correlation performed regarding bias, becomes close of 1,00 and p value becomes higher than 0,05 for meaning that two-tailed p value does not cause a meaningful difference.It has been determined that there is not publication bias in the study since it has been found that Tau coefficient was 0,11667 and two-tailed p-value was 0,528. No statistically meaningful publication bias could be determined in Egger regression analysis (intercept=0,99821, t (16) = 0,834, 2-tailed p=0,417).

Figure 3. *Funnel Plot of Publication Bias by Marital Status*



It has been determined that there was no publication bias in the study since Tau coefficient was found 0,263 and two-tailed p-value was found as 0,188.No publication bias statistically meaningful has been determined in Egger regression analysis (intercept=-1,72577, t (12) = 2,744, 2-tailed p=0,177).

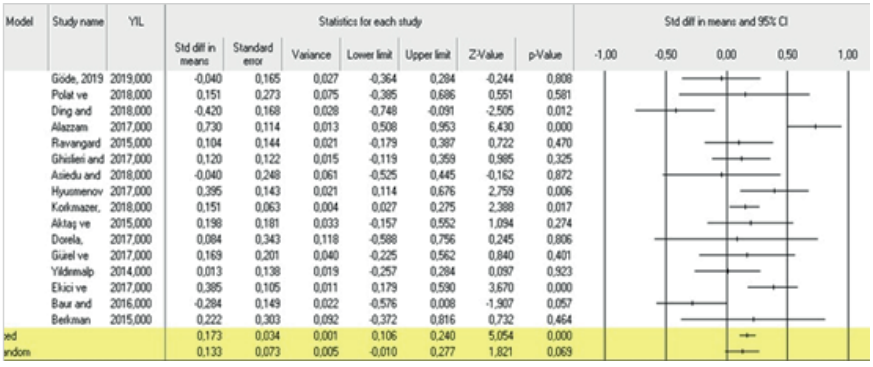
3.2.Effect Model, Effect Size and Heterogeneity Test of the Studies

If the studies are homogenous, fixed effect model should be taken into account, if it is heterogeneous, random effects model's value should be taken into account (Dinçer 2014:19).

3.2.1.Effect sizes of the studies according to gender variable

The number of studies containing sufficient data, for being included into meta-analysis and examining relationship between gender variable and work-family conflict, is 16. As a result of analysis on 16 studies included into meta-analysis in accordance with gender variable, Q value is 56,883, df15 and p values are 0,00. It has been found out that there is a meaningful difference between the studies and that the studies are heterogeneous due to the fact that p value is lower than 0,05. Considering that the studies included into meta-analysis for gender variable, are heterogeneous, random effects model's value has taken into account. Minimum value of average effect size in random effect model (Hedges's g) is - 0,010 and its maximum value is 0,277. Average of effect size is 0,133 (s.s=0,073). In this case, it has been reached a conclusion that gender has a positive effect on work-family conflict experienced, that female nurses experience more work-family conflict compared to male nurses. In other words, it can be said that being female nurse cause experiencing more work-family conflict then male ones(Figure 4).

Figure 4. *Effect size according to gender variable*

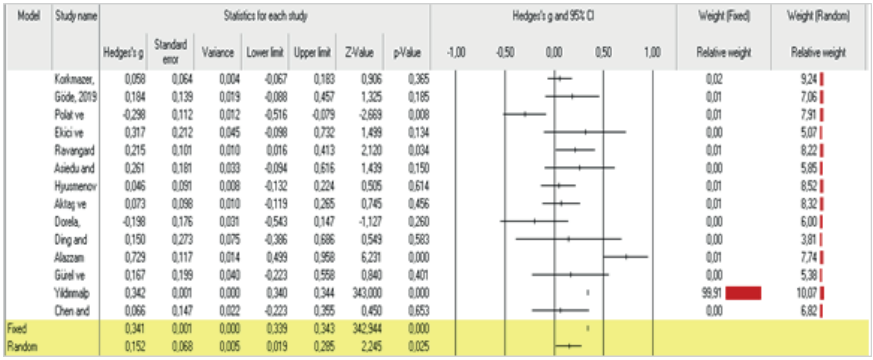


3.2.2. Effect size based on marital status

In analysis result belonging to 14 studies included to meta-analysis according to marital status variable, Q value is 98,725, df13 and p values are 0,0000. It has found that there is a meaningful difference between the studies due to the fact that p value is lower than 0,05, therefore, that the studies are heterogeneous. Minimum value of average effect size in random effect model (Hedges's g) is 0,019 and its maximum value is 0,285.

Average of its effect size is (s.s=0,068).In this case, it has been concluded that marital status has a weak positive effect on work-family conflict. In other words, it can be said that being married has a weak positive effect in experiencing work-family conflict compared to being single. Married nurses experience more work-family conflict compared to single ones (Figure 5).

Figure 5. *Effect size according to gender variable*



3.3. Sub-Group Analyses (ANOVA Analyses)

In the studies included into meta-analysis, 4 categorical variables have been defined in total; publication type, institution where the study is made, country where the study is made and work-family conflict scale used in the study and sub-group analyses have been made with these variables.

As a result of moderator analysis performed according to gender variable, it has been found that publication type (p=0,707), institution type of the study (p=0,250) and work-family conflict scales used in the studies, are not moderators. When the countries where the studies included into meta-analysis were made (p=0,000), it has been determined that they are moderators. When the countries where the studies were made, it has been found that female nurses working in Jordan experience more work-family conflict and it has a strong effect on them.On the other hand, it has been found that nurses working in Turkey experience more work-family conflict, however it has a weak effect.

As a result of moderator analysis performed according marital status variable, it has been found that publication type (p= 0,111), institution type where the study is performed (p=0,767) and work-family conflict scales used in the studies (p=0,535), are not moderators. When the countries included into meta-analysis are taken into account (p=0,000), it has

been determined that they are moderators. When the countries where the studies were performed are taken into account, it has been seen that married nurses living in Jordan experience the most work-family conflict and it has a strong effect on general effect size. On the other hand, it can be said that married nurses working in Turkey experience more work-family conflict and it has medium effect on general effect size.

Result and Discussion

As a result of literature review performed between 2009 and 2019, 17 studies examining gender and marital status of nurses on their work-family conflict, were added to meta-analysis. The total sample size is 6536. In the study performed on nurses by Polat et. al (2018), it was concluded that being female and male for nurses and difference of their education status have no effect on work-family conflict (p>0,005); however, regarding marital status variable, single nurses experience more work-family conflict sourced from higher work life than married nurses. In the studies performed by Karatepe and Kılıç(2005) and Bragger et. al. (2005), it was concluded that when it is a married person, there is more work-family conflict.

In the study performed by Korkmazer (2018) on 995 nurses, it was found that males experience more work-family conflict compared to females, in term of their marital status, there is not difference between being married or single. In the studies performed by Cinamon (2006) and Zincirkıran (2013), on the contrary, it was found that females experience more work-family conflict than males, they are less successful managing the conflict compared to males.In the study performed by Sönmez et. al. (2015) on nurses, it was concluded that there is no effect of family area of nurses who are single or divorced, on their role requirement in their works, family life of married nurses is affected from their role requirements for their work.

In the study performed by Hyusmenova (2017) on 521 nurses, female and married nurses experience higher level of work-family conflict than the ones who have child. In the study performed by Ding et. al. (2018) on 487 nurses, it was concluded that male nurses experience more work-family conflict than females, nurses who are single and have no child, have lower work satisfaction.In the study performed by Göde (2019) on 227 nurses, it was determined that there is no meaningful difference statistically between work-family conflict and gender and marital status variable for nurses.In the study performed by Gencer (2017) related to healthcare professionals, it was found that there is a meaningful difference between size of conflict from work to family and genders for the professionals, meanwhile, there is no meaningful difference statistically between gender

and size of conflict from family to work. It was found that there is no meaningful difference statistically for nurses between their marital status and work-family conflict and size of conflict from work to family. In the related study of Korkmazer (2018), it was found that there is no meaningful difference between work-family conflict and marital status of the professionals.

In the study performed by Alazzam et.al. (2017), it was found that nurses experience work-family conflict, it is negatively related with age and positively related with being female and having no child care facilities in work place cause positive effects for arising a conflict from work to family.In the study performed by Ravangardve et al. (2015) on 400 nurses, it was concluded that female and married nurses experience higher work-family conflict. In the study performed by Ghisleri et. al.(2007) on 500 nurses, it was found that female nurses experience more work-family conflict than male ones and in the study performed by Baur et.al (2018) on 48 nurses, it was found that there is effect of gender variable on work-family conflict, in the study performed by Berkman(2015) on 1524 nurses, it was found that being female and married and also having a small child at home increase work-family conflict.

In study performed by Xhako (2017) on 164 nurses, it was concluded that gender and marital status variables have effect on work-family conflict. In the study performed by Ekici et. al. (2017) on 94 nurses, it was concluded that married nurses experience more work-family conflict compared to single nurses. It is seen that married nurses experience work-family conflict as a result of the study performed by Gürel et. al. (2017) on 103 nurses.In the study performed by Chen et.al.(2015) on 186 nurses, it was found that married nurses experience more work-family conflict compared to single ones. In the study performed by Asiedu et.al. (2018) on 134 nurses, it was concluded that long working hours and weekend works, being female, being married, having an elderly in need of nursing have positive effect on work-family conflict that nurses experience.

As a result, it has been concluded that gender and marital status of nurses, have effect on having work-family conflict for nurses. It is seen that female and married nurses working in institutions experience more work-family conflict.According to meta-analysis result, it has been concluded that female nurses experience more work-family conflict than male nurses, on the other hand, married nurses experience more work-family conflict than single nurses. In other words, it can be said that gender and marital status variables have effect on having work-family conflict.

Considering the research results, in order to prevent or decrease experienced work-family conflict, working models practiced in other countries

can be preferred. These models can create time and environment for performing roles and responsibilities between work and family. For instance, it is suggested to practice a wages policy based on working preference by planning weekly/daily working hours of nurses based on institutional policy (working 50%-75% of it, full time working, shift working, only daytime/night working, night and weekend working, etc.).

Due to the fact that experienced status as a result of work-family conflict also affects institution, it is suggested to increase administrator supports and to plan family friendly practices related to individuals experiencing the conflict such as interview with institution psychiatrist, kindergarten support, flexible working hours, having job definitions, arrangement in working hours, arrangement in working types (allowing to work in day shit if the person works in a mixed shift in day and night).

In order to make thesis and studies accessible taking place in the literature in meta-analysis, to make studies that were performed wrong by researchers and not limiting them by authors, and to include performed studies into meta-analysis, it is suggested to statistical data such as mean, sd, n, p, X2 etc., related to study, in findings section in detail.

Conflict of Interest Statement: We have no conflicts of interest

Funding Statement: We did not use any funding for the study.

Implications for Nursing Management: It is important to reduce work-family conflict have applications to managers support employees, flexible work schedules in institutions, etc.

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The references marked with an asterisk (*) indicate the studies included in the meta-analysis.

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Chapter 19

MOLECULAR DOCKING STUDIES OF ZN(II) AND MN(II) COMPLEXES OF 6-BROMOPICOLINATE AS A-GLUCOSIDASE AGENTS

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INTRODUCTION

Diabetes mellitus (DM) consists of two types and they are most common metabolic diseases all over the world. Type 1 diabetes is caused by insulin deficiency and constitutes 5-10% of the diabetic population. Type 2 diabetes (T2DM) is known as one of the most widespread metabolic degenerative diseases today and one of common cause of T'DM is insulin resistance (Ghani, 2015:133). T2DM drugs can be divided into the following seven groups according to their mechanism of action; insulin secretagogues, α-glucosidase inhibitors, aldose reductase (AR) inhibitors, simulation insulin sensitizers, insulin drugs, and traditional Chinese medicine (Wang et al., 2012:2119).

α-Glucosidase inhibitors (AGIs) hold up carbohydrate digestion and extend the glucose absorption and carbohydrate digestion time (Demadis and Katarachia, 2004:627). Only a few AGIs are commercially available and cannot meet the need due to the side effects they show (Ghani, 2015:133). Consequently, the design and synthesis of specific glucosidase inhibitors are interested by scientist.

In the literature, various heterocyclic molecules containing nitrogen atoms such as indole, pyrrolidine, piperidine inhibit the α-glucosidase enzyme (Mao, 2007:1493). It is stated to be used in the treatment of hyperglycemia in T2DM. Synthesis and investigation of biochemical characters of pyridine derivative ligands such as picolinic acid is important because of the many applications of these ligands in ion exchange, catalysis, luminescence, nonlinear optics and materials chemistry, as well as synthesizing different structures including coordination complexes (Monot et al., 2008:6243; Hardy and La Duca, 2009:308; Umeda et al., 2010:5882). Some picolinic acid complexes have been considered in many design and synthesis studies due to their anti-diabetic activity (Avci et al., 2018:7198 and 2019:747; Altürk et al., 2019:1265).

Molecular docking studies are widely used to design novel effective alternative molecules against many known diseases. In this chapter, 6-bromopicolinate and its Zn(II) and Mn(II) complexes were selected as the target potential α-glucosidase inhibitors. The AutoDock4 program was applied to determine their interactions between α-glucosidase enzyme and selected inhibitors.

METHODS

The α-glucosidase (S. cerevisiae isomaltase (PDBID: 3A4A)) was chosen rigidly and molecular docking was applied with the AutoDock4 program implemented via the graphical user interface AutoDockTools (ADT1.4.6) (Morris et al., 1998:1639). All calculations for inhibitors-tar-

get enzyme insertion were performed using the Lamarckian genetic algorithm method (Solis and Wets, 1981:19). 2D and 3D structures demonstrating the interactions between α-glucosidase and inhibitors were drawn in Discovery Studio 4.0 (Systèmes, 2016).

DISCUSSIONS

The enzyme-inhibitor interactions, distance values and calculated inhibition constants (Ki) for 6-bromopicolinate and its Zn(II) and Mn(II) complexes are given in Table 1. According to molecular docking results, 6-bromopicolinate and its Zn(II) and Mn(II) complexes exhibited the α-glucosidase inhibitory activity. The calculated inhibition constants (Ki) values ranged from 71.15 μM to 1161.0 μM.

6-bromopicolinate was found to be moderate α-glucosidase inhibitor with the Ki value of 1161.0 μM. On the other hand, Zn(II) complex of 6-bromopicolinate showed the strongest α-glucosidase inhibition with the Ki value of 71.15 μM, while its Mn(II) complex had inhibitory activity against α-glucosidase with the Ki value of 136.72 μM.

Tablo 1: *Enzyme-inhibitor interactions, distance values and calculated inhibition constants (Ki) for 6-bromopicolinate and its Zn(II) and Mn(II) complexes*

Substrate	Receptor	Interaction	Distance (Å)	Ki (mM)
6-Br-picH				1161.0
-C=O	LYS-45	Convent. H-Bond	3.05	
-COO-	LYS-45	Convent. H-Bond	2.95	
Pyr	GLU-47	Amide Pi-Stacked	4.13	
-Br	ILE-485	Alkyl	4.58	
-Br	TYR-48	Pi-Alkyl	4.78	
Zn(II) complex				71.15
H2O	TYR-566	Convent. H-Bond	2.32	
H2O	GLY-564	Convent. H-Bond	2.67	
H2O	GLY-564	Convent. H-Bond	2.75	
-C=O	LYS-373	Convent. H-Bond	3.05	
Pyr	LYS-568	Pi-Donor H-Bond	4.17	
Pyr	PRO-567	Pi-Alkyl	4.46	
Mn(II) complex				136.72
H2O	SER-162	Convent. H-Bond	2.27	
-COO-	SER-162	Convent. H-Bond	3.11	
-Br	THR-165	Carbon H-Bond	3.54	
Pyr	ALA-418	Pi-Alkyl	4.90	

It can be shown the structure–activity relationship (SAR) from Table 1: (i) the selected target molecules had the α-glucosidase inhibitory activity; (ii) both complexes of 6-bromopicolinate exhibited stronger inhibitory activity against α-glucosidase than 6-bromopicolinate; (iii) the Ki values of Zn(II) and Mn(II) complexes were 71.15 µM and 136.72 µM, respectively, which are 16.3-fold and 8.5-fold active than 6-bromopicolinate (Ki = 1161.0 µM); (iv) The fact that the α-glucosidase inhibition value of the Zn complex is lower than that of the Mn complex may be explained that the atomic diameter of the Zn metal is larger than that of the Mn metal and that the Zn complex is bulkier than Mn complex.

According to the molecular docking results (Table 1), various interactions such as H-bonds and different pi-stacking were observed between the pyridine rings, carboxyl groups, and H2O, involved in the complex coordination, and amino acid residues of α-glucosidase.

2D and 3D structures showing the interactions between enzyme active site and 6-bromopicolinate and its Zn(II) and Mn(II) complexes are given Figures 1-3, respectively.

According to the molecular docking results of 6-bromopicolinate, conventional H-bond between hydroxyl group/carbonyl group and LYS-45 (2.95 Å/3.05 Å), and pi-alkyl/alkyl interactions between –Br atom and TYR-48(4.78 Å)/ILE-485(4.58 Å) as well as amide pi-stacked interaction between pyridine ring and GLU-47(4.13 Å) were detected (Figure 1).

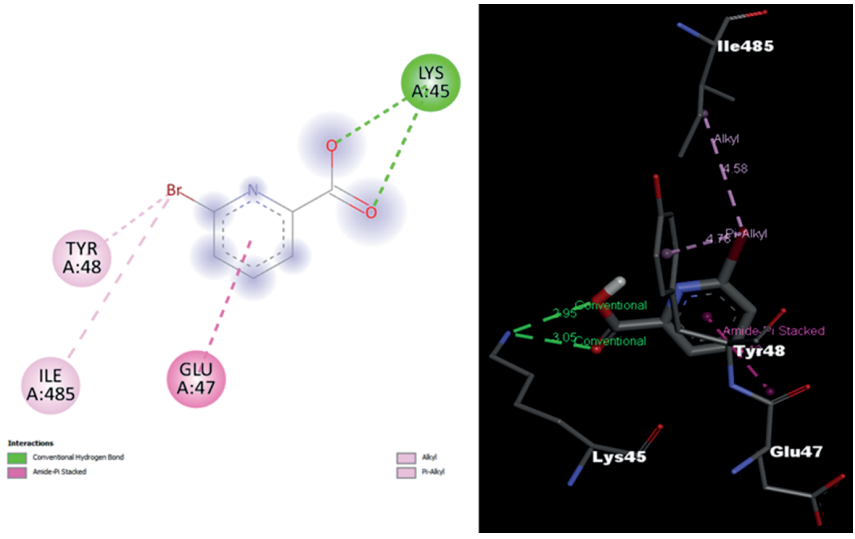

Figure 1: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and 6-Bromopicolinate*

By considering the molecular docking results of the Zn(II) complex of 6-bromopicolinate, it was observed the conventional H-bond between the water ligand/carbonyl group and TYR-566(2.32 Å), GLY-564(2.67 Å)/LYS-373(3.05 Å), and pi-donor/pi-alkyl interactions between pyridine ring and LYS-568(4.17 Å)/PRO-567(4.46 Å) (Figure 2).

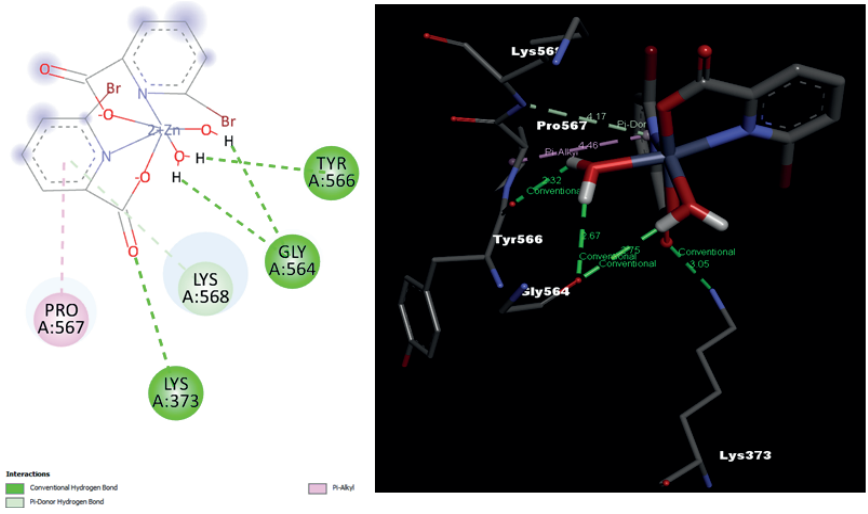

Figure 2: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and Zn(II) Complex of 6-Bromopicolinate*

The molecular docking results of the Mn(II) complex of 6-bromopicolinate demonstrated the conventional H-bond between water ligand/carboxylate group and SER-162(2.27 Å/3.11 Å), carbon H-bond between –Br atom and THR-165(3.54 Å), and pi-alkyl interactions between pyridine ring and ALA-418(4.90 Å) (Figure. 3).

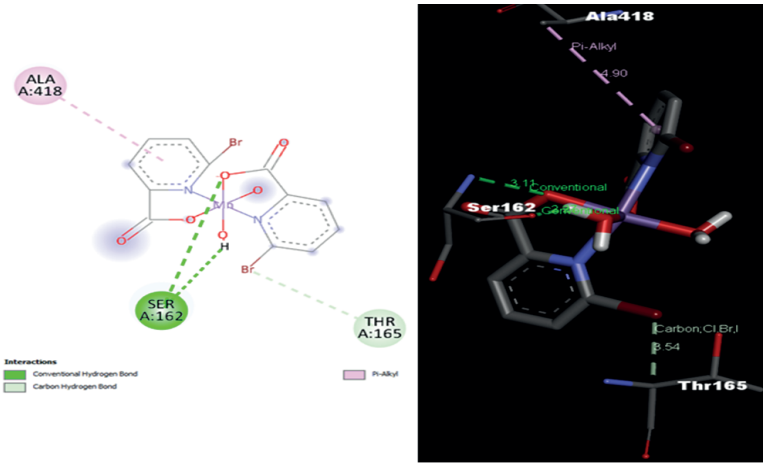

Figure 3: *2D and 3D Structures Showing the Interactions Between α-Glucosidase and Mn(II) Complex of 6-Bromopicolinate*

CONCLUSION

In conclusion, according to molecular docking studies, the 6-bromopicolinate and its Zn(II) and Mn(II) complexes have α-glucosidase inhibitory activity. Due to their various interactions with amino acid residues in the enzyme active site, the synthesis and design of novel metal complexes consisting of some halogens such as -Cl and -Br, and electron-withdrawing group such as -NO2 can be considered.

It is clear that the predicted inhibition values of complex structures are lower than those of the ligand, which will allow synthesis and design studies of new complex structures.

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Chapter 20

LAPAROSCOPIC TOTAL EXTRAPERITONEAL REPAIR OF INGUINAL HERNIA (TEP)

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Hernia, in general, is the displacement of any organ or tissue in the body from where it should normally be to another anatomical region. When a hernia is mentioned, an inguinal hernia or umbilical hernia usually comes to mind, but it can be almost any organ hernia. Inguinal hernia is one of the most common diseases that general surgeons encounter. The displaced organ in an inguinal hernia is usually a part of the intestines in the abdomen. Here, the intestines move through the abdomen, passing through the abdominal wall, and settle in the subcutaneous region. This may only be the result of weakness or rupture in the muscles of the abdominal wall, which is a solid structure. It usually manifests as a raised swelling from the skin which, increases while standing, and becomes indistinct when lying down.

Hernias are examined in two main groups:

1- Internal Hernias (Internal Hernias): Internal hernias occur inside the body and cannot be seen from the outside. They are formed due to displacement of internal organs inside the body. For example, gastric hernia (hiatal hernia), diaphragmatic hernia (diaphragmatic hernia), and internal hernia of the small intestines. In gastric hernia, the first parts of the stomach, called the cardia and fundus, become hernia by passing upwards into the thoracic cavity as a result of the expansion of the muscle opening called hiatus and entering the esophagus from the thoracic cavity into the abdominal cavity by passing through it. Although there is no visible swelling, the patient has complaints such as sour water in the mouth, pain, and a burning sensation spreading upwards along the stomach and esophagus. In diaphragmatic hernia, as a result of damage to the muscle called the diaphragm, which separates the thoracic cavity and the abdominal cavity, abdominal organs such as the stomach, spleen, large intestine,and small intestine enter the chest cavity. Again, there is no visible swelling, but there is a wide range of complaints from digestive problems to severe respiratory distress. In internal hernias of the small intestines, the intestines may enter into some small anatomical spaces in the abdomen or some areas formed due to adhesions and get stuck there. In this case, signs of intestinal obstruction occur.

2-External Hernias (External Hernias): External hernias are the hernias that are reflected in the visible external part of the body. The swelling caused by the internal organs is seen as swelling under the skin. External hernias occur in three main regions; anterior abdominal wall (ventral hernias), posterior abdominal wall, and perineum. The hernias in the last two regions are very rare and difficult to diagnose. Ventral hernias, on the other hand, are very common hernias ;as examples of them we can show inguinal hernias (Inguinal Hernia), umbilical hernias (umbilical hernia), incisional hernias (incisional hernias), epigastric hernias and Spiegel her-

nias. Inguinal hernias are the most common hernias. Hernias that develop in both inguinal regions are called inguinal hernias. It can be detected during a doctor's examination performed for another reason, without giving any complaints, or it can manifest itself with some complaints. There may be local pain in the inguinal region; this is usually seen in the initial stage and is related to the enlargement of the inner opening of the inguinal canal through which the hernia passes.

Pain may be felt around the umbilicus; because the fat mass called the omentum in the abdomen is stretched by entering the hernia area. There may be a feeling of discomfort in the groin area, which is due to the intestines coming in and out of the hernia sac. There may be visible swelling in the hernia area. This swelling enlarges when standing, coughing or straining, and shrinks or disappears when lying down. Sometimes, this swelling can be inserted not by itself, but only by pushing by hand. They are intra-abdominal organs that cause swelling and have entered the hernia sac. If the tissue that has entered the hernia is the intestine, there may be a crackling sensation under the fingers when touched by a hand.

Inguinal hernia may occur due to obesity, heavy exercise, chronic cough, chronic constipation, prostate disease, diseases with acid (fluid collection) in the abdomen, pregnancy , and tumors of the pelvic cavity, as well as congenital (congenital) origin.

There are three types of inguinal hernia; indirect inguinal hernia, direct inguinal hernia and femoral hernia. An indirect inguinal hernia is a hernia formed by passing through the anatomical structure called the inguinal canal; there is usually a congenital weakness. It is the most common hernia type, and can reach to the scrotum in advanced stages. Direct inguinal hernia occurs due to the weakness of the region inside the inguinal canal; it is more common in men, and the possibility of strangulation is less according to other types of inguinal hernias. It usually occurs due to later causes. These two types of hernias form in the upper part of the ligament, called the inguinal ligament. On the other hand, a femoral hernia occurs under this ligament, passing through the space called the femoral canal, which is more common in women, and is the type of inguinal hernia with the highest probability of strangulation.

The diagnosis of an inguinal hernia can be made by palpation. There is a special method of examination with fingers. Even the type of hernia can be understood with this method. If the diagnosis cannot be made by manual examination, it can be easily diagnosed with ultrasound or computed tomography.

The treatment of inguinal hernia is surgery. It is one of the most frequently performed surgeries in the world. Today, there are several very

modern and effective surgical methods. The oldest evidence of an inguinal hernia was found in ancient Egypt in 1552 BC.[1],[2] It has not been easy to reach this point in this disease, which has been known for about 3500 years and has been tried to be treated surgically for about 2000 years. Because it took many years to understand the anatomy of the inguinal region and the development of the dissection technique was expected for this. In addition, it took a long time for the concept of antisepsis to enter surgery. As a result of the fulfillment of the above conditions about 130 years ago, the types of herniorrhaphy surgery that Bassini initiated with the principle of anatomical tissue repair became widespread.[3],[4] However, there was a substantial problem of recurrence here. It took 70 years for the hernioplasty type surgery to reduce this recurrence problem and to strengthen the abdominal wall without creating tension. Thanks to the likes of Lichtenstein and Stoppa, methods of strengthening the abdominal wall with mesh developed, and the problem of recurrence began to be overcome.[5],[6] However, in some methods, morbidity rates were not low due to large incisions and extensive dissections. Since the 1980s, the use of laparoscopy began to enter hernia surgery, thus achieving the current successful situation. Recurrence and morbidity rates have decreased considerably. Laparoscopic methods have also contributed significantly to shortening of postoperative recovery period.

P.Fletcher first used the laparoscope in 1979 for inguinal hernia repair.[7] Later, Ralph Ger [8] in 1982, S.Bogojavalensky [9] in 1989, L. Schultz [10] in 1990, M.E.Arregui [11] in 1992 published their experience with the TAPP technique.

GS.Ferzli [12] in 1992, JM.Himpens [13] in 1992, JB.McKernan and HL.Laws [14] in 1993 and EH.Phillips [15] in 1993 published their experiences with the TEP technique.

Recurrence rates are not zero in laparoscopic inguinal hernia surgery. Here, the surgeon's experience in laparoscopy, especially in laparoscopic hernia surgery, is as important as the patient's factors. For example, a surgeon who can successfully perform a laparoscopic cholecystectomy may not have the same success in laparoscopic inguinal hernia surgery. In addition, choosing the appropriate laparoscopic method for the patient is an essential factor that increases success.

The laparoscopic inguinal hernia surgeries used today are the intraperitoneal onlay patch approach, the transabdominal preperitoneal approach (TAPP) and the total extraperitoneal approach (TEP). Of these, TAPP and TEP are the two most commonly used methods.

With TEP, which is the method we use more frequently, approximately 1000 patients were operated on in our center for 15 years, and very low

recurrence rates (< 1%) were found. The indications, contraindications, surgical technique, and possible complications of this method will be explained in light of our experience.

In order to determine which type of surgery will be chosen for which patient, the surgeon must first have knowledge of both commonly used methods (TEP and TAPP) and be experienced in these surgeries. Otherwise, he will be inclined to choose the method with which he is more experienced. This may be the reason for the wrong choice. The other factor in the selection to be made is the characteristics of the patient. The type of hernia in the patient, whether the event is urgent or elective, the patient's history and previous surgeries, and whether the hernia is a recurrence are the issues that should be questioned.

Our preference is usually TEP if there is no obligation imposed by the above conditions. Working in the preperitoneal area without entering the peritoneum reduces the chance of intra-abdominal organ injury. Since the trocars do not pass through all the abdominal layers and remain in the preperitoneal area, the possibility of hernia formation from the trocar incisions is very low. Since the peritoneal closure process, which is the last stage in the TAPP method, is not required in the TEP method, the surgery takes less time. Since it is not studied in the abdomen, the possibility of some complications, such as brid ileus in the postoperative period, is low.

In some cases, TAPP is a more reliable surgery. Since the inside of the hernia sac can be seen directly, it provides a safer working environment for the surgeon in strangulated or sliding hernias. The strangulated intestinal segment can be evaluated definitively in terms of the vitality of the intestine and it can be determined more accurately whether resection is required in the reduced part. In addition, it provides a safe reduction. Lower abdominal pain can be caused by many factors, especially in women. For this reason, TAPP may be a reason for preference in female patients with inguinal hernia and lower abdominal pain because diseases that can cause pain, such as endometriosis or ovarian pathologies, are not missed due to direct observation of the inside of the abdomen.

Laparoscopic approach is quite ideal in recurrent hernias if the previous operation was performed as an anterior intervention. The posterior wall is clearly visible with the laparoscopic approach, so the pre-placed mesh is not a challenge.

As in all laparoscopic surgeries, TEP is performed under general anesthesia. Preoperative routine examinations are performed in a way that is not different from other surgeries. The patient is prepared after a blood count, biochemical tests, chest X-ray, ECG, and serology tests are done. If necessary, consultations are made with the relevant branches, and addi-

tional examinations that may be requested are carried out. An anesthesiologist makes an evaluation. If the patient uses blood thinners, precautions are taken. The surgeon visits the patient in his room before the operation and marks the operation area and side. Because the working area is very close to the bladder, a urinary catheter is inserted into the patient just before the operation and removed after the operation but before he wakes up.

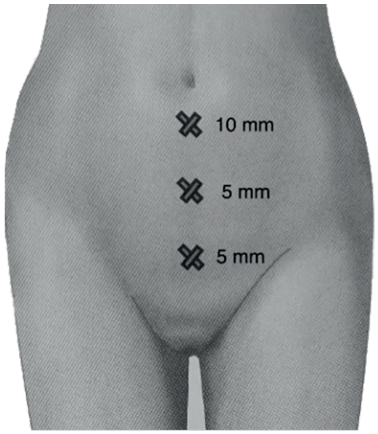
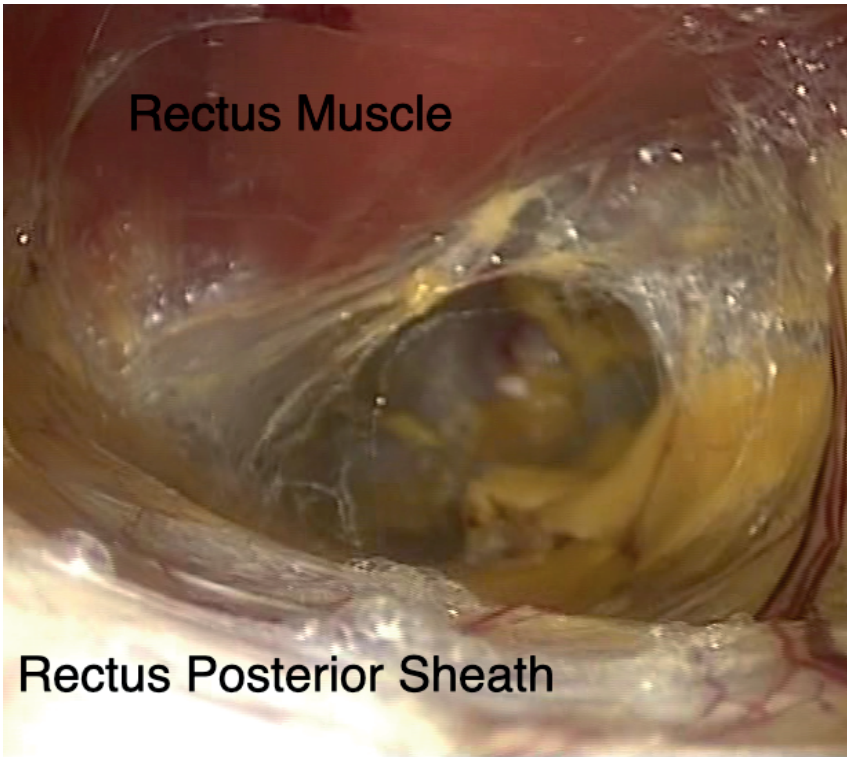


Figure 1: Trocar entry points in TEP method

We use a total of 3 trocars in TEP surgery. One of them is 10 mm in diameter, placed just below the navel and directed to the preperitoneal region. The other two are 5 mm in diameter and are inserted into the pre-peritoneal area by placing them 5 cm apart on the sub-umbilical median line. (Picture 1)

For a 10-inch trocar, the incision is made 1 cm below the navel. If the operation is to be performed unilaterally, the incision is a 1 cm incision extending from the midline to the operation side. If the operation is to be performed bilaterally, the incision is extended towards the side where the hernia is larger. The skin and subcutenaus tissues are cut under the direct vision. The anterior sheath of the rectus muscle is cut transversely. The lower sheath is made visible by pulling the muscle laterally with a retractor. The trocar is advanced towards the pubis along the midline between the posterior surface of the rectus muscle and the lower sheath. The trocar is inserted with the chuck removed. Several skin sutures are placed to prevent air from escaping into the incision through which this trocar passes. The threads of one of these sutures are left long and passed through the hole at the end of the trocar and held with a hemostat. In this way, we have the opportunity to move our trocar back and forth when necessary during the surgery and fix it.



Picture 2: Opening the operation area

There are two options for the creation of the preperitoneal space to be operated. One is to open this area by inflating the balloon using a balloon trocar. However, we do not prefer this method, because we there is a possibility of injury and bleeding in the area, even if the balloon is weak during inflation. We prefer to use the second option for opening the surgical field. In other words, after connecting the CO2 gas to the 10 mm trocar, we open the area ourselves with 0 0 optics, by seeing the tissues in front of the telescope through the monitor, with back and forth and slight right-left movements. We think this method is safer. (Picture 2)

The first thing we pay attention to during the creation of the preperitoneal surgical field is to try to see the inferior epigastric vessels. In this way, we secure a vessel whose hemostasis can be very difficult in case of bleeding, and determine a critical reference point for the surgery to be performed. (Picture 3)

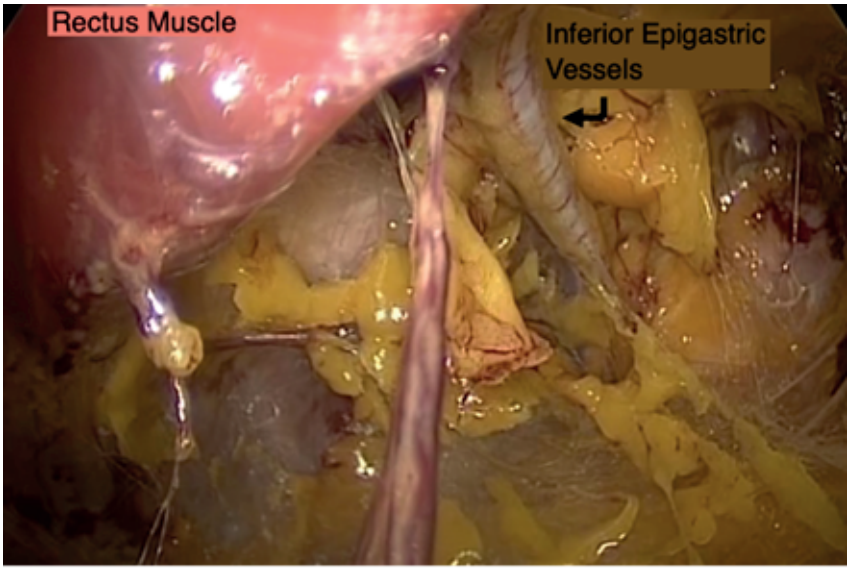


Figure 3: Visualization of the epigastric vessel

After creating enough space, two trocars of 5 mm are placed in the preperitoneal space along the midline. Before placing these trocars, observation is made from the monitor by entering the area vertically with a syringe needle from the place where the trocar will be inserted. In this way, trocar entrances are tried to be made more secure. We think that trocar entries made into the preperitoneal area are a little more dangerous than the entries made into the intraperitoneal area.

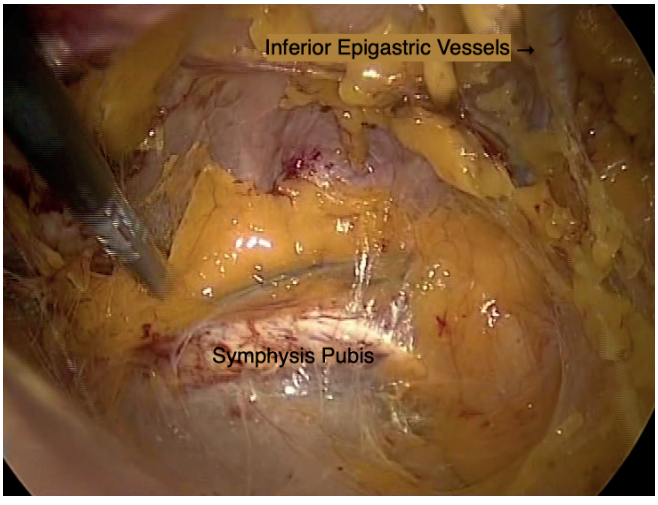


Figure 4: Exposure of the pubis

The next step is to reveal another reference point, the pubic bone. This area is important for the mesh to be placed and is where the medial edge of the mesh is attached. It is necessary to be very careful not to bleed the corona mortis during the exposure of the pubis. This vessel, like the inferior epigastric vessel, is a difficult structure to hemostasis. (Picture 4)

Another reference point is the lateral wall of the abdomen. By entering this area and revealing the wall, the position and shape of the indirect hernia sac become easier to understand. The lateral edge of the mesh is attached to the lateral wall of the abdomen. The most crucial point to be considered during this process should not be to coincide with the nerves because the pain triangle is located in this region. Mesh fixing should be done as much as possible on the upper part of the lateral wall. (Picture 5)

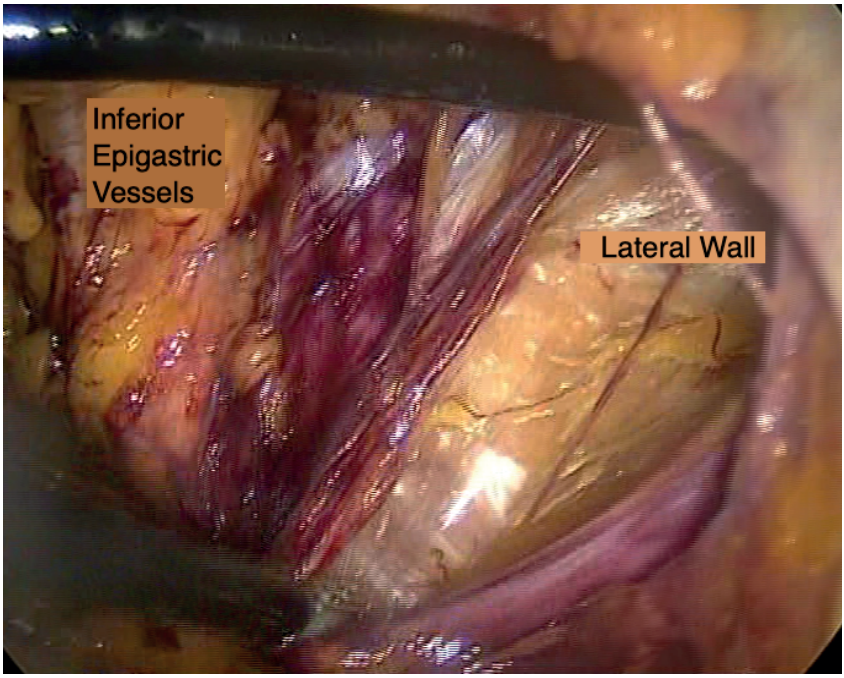


Figure 5: Opening of the lateral wall

The laparoscopic instruments we use in the TEP method are not much different from those used in other laparoscopic surgeries. We usually perform tissue dissection with two graspers. We think it would be dangerous to use dissector or scissors here. Dissector is generally preferred during hemostasis and scissors are the tools we prefer for cutting cauterized tissues. If there is an opening in the peritoneum, we use clips to close it. A tucker is used to fix the patch. The clinch is used during the insertion of the patch through the 10 mm trocar.

After establishing these three reference points and completely liberating the preperitoneal area, it is time to find and reduce the hernia defect and hernia sac. With the TEP method, all three types of inguinal hernias can be repaired. In other words, it is possible to cover direct, indirect and femoral hernia defects with mesh. Indirect hernia defect is located lateral to the inferior epigastric vessels, direct hernia defect is located medial to the inferior epigastric vessels and above the inguinal ligament, and femoral hernia defect is located medial to the inferior epigastric vessels and below the inguinal ligament. Indirect and direct hernia defects can be found together in the same patient. There are two areas that need attention during dissection. The first of these is the area called the pain triangle, which should not be touched during the dissection and during the fixation of the mesh, otherwise the complaint of pain in the postoperative period would be too much. In some rare cases, it may even be necessary to re-operate the patient and remove the stapler due to the pain caused by the stapler hitting these nerves. The second important region is the region of large vessels with high mortality if injuries occur. The iliac and femoral arteries and veins are located in this region. (Picture 6)

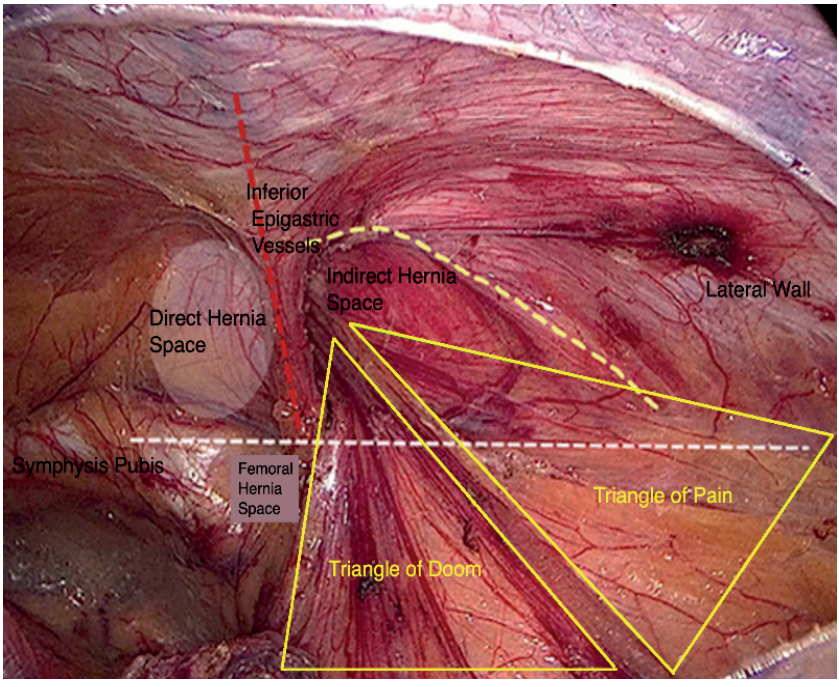


Figure 6: General view of the inguinal region

Dissection of direct hernia sac is relatively easy compared to indirect hernia. It lies just medial to the inferior epigastric vessels. The defect may be in the form of a vague cambering, or it may be in the form of a rather

large cavity and wide mouth. After the contents of the sac are carefully reduced, if a large and deep cavity remains, the transverse fascia forming the wall of this cavity should be pulled with a tool and fixed to the pubis bone. In this way, the defect surface is flattened and it is tried to prevent fluid accumulation and infection in the postoperative period. The mesh is placed on it to cover the direct hernia defect, which also includes the area that may be an indirect hernia.

Dissection and reduction of the indirect hernia sac is a more difficult procedure. In an indirect hernia, the sac is located anterolaterally to the cord elements. Testicular vessels and vas deferens should be protected from injury. (Picture 7) There may be a sliding or suffocated bowel section in the pouch, and you must be very careful not to damage them. Reducing the sac in scrotal hernias is a very laborious task. The reduction of the hernia sac should be continued to a level that will ensure that it is not under the mesh to be placed. Otherwise, a part of the intestines may enter under the mesh and cause early recurrences. In some cases where there is a lot of adhesion, the peritoneum can be opened during dissection, this opening should be closed with a clip without allowing it to grow too much. Sometimes there is a structure called cord lipoma inside the hernia canal, this structure is located lateral to the hernia sac and spermatic cord. We usually prefer to reduce this, because otherwise, if the patient feels the lipoma with his hand in the postoperative period, he may think that the hernia is not repaired.

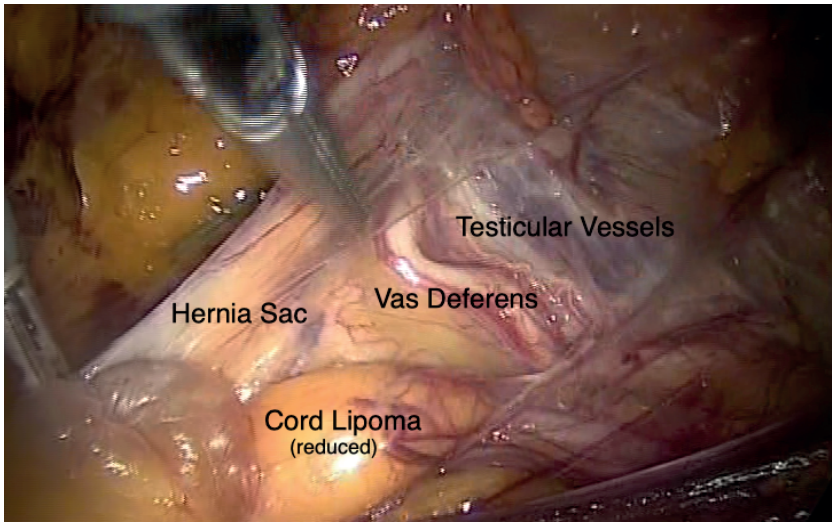


Figure 7: Indirect hernia

After all, hernias are reduced, and hemostasis is controlled, it is time to place the patch. The patches we use are polypropylene. (Picture 8) These

are the types that we usually prefer, called 3D. Because it is effortless to control and place them in a very narrow space. The patch is held at one end with an instrument and inserted into the preperitoneal region through a 10 mm trocar under the umbilicus. The patch is then manipulated by inserting the camera back in and extending to cover all hernia defects.

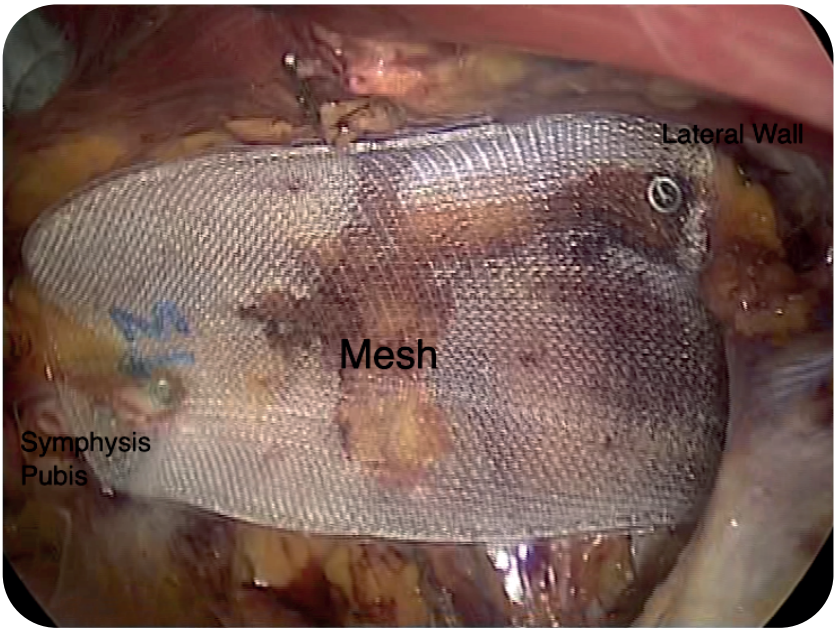
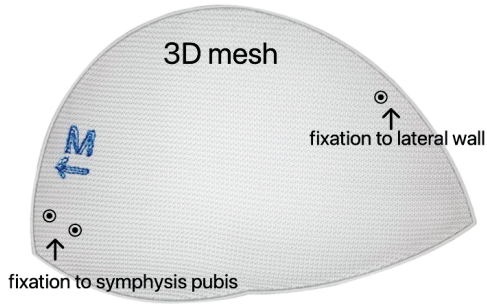


Figure 8: Polypropylene mesh

The place where the patch will coincide with the pubis is pre-marked (the letter M in picture 9) and this is first fixed to the symphysis pubis with a stapler. The patch is stretched sideways so that there is no slack and stapled again to a place on the lateral wall above the pain area. We then prefer to put a second staple on the pubis. (Picture 9) It is checked that the reduced parts are not under the patch. If the testicular vessels and spermatic cord are holding the patch in the lower part, this swelling is removed by making a small incision on the lower edge of the patch.



Picture 9: 3D patch

After the last check of hemostasis , the 5 mm trocars are pulled in sequence without evacuating the air inside, and it is checked whether there is bleeding at the trocar entry sites. By starting to evacuate the air without pulling the camera, it is observed how the preperitoneal space is closed and how the patch is placed. After the air is evacuated, the 10 mm trocar is also removed. The upper fascia here is sutured and after the skin is sutured to all incisions, the patient's urinary catheter is removed and the surgery is terminated. Although it varies according to the condition of the surgery, we do not insert a drain to a large extent.

We usually discharge our patients the day after the surgery. Rarely, our patients who had surgery in the morning were discharged the same day. There is no obvious restriction after the operation, after a few hours of follow-up, mobilization is ensured during the day as much as the pain allows. After discharge, daily activities are allowed. The majority of patients return to their routine life in less than a week.

Complications we see are wound infection, seroma at the surgical site, and recurrence. In our series, their total rate is less than 1%. Wound infections resolved with conservative treatments without causing serious problems. Seroma resolves spontaneously within a few weeks. We did not have any patients who needed to be aspirated.

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Chapter 21

AUDIOLOGIC ASSESSMENT IN CHILDREN WITH HEARING LOSS

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Introduction

Hearing loss that occurs in congenital or first years of life negatively affects all areas of development, especially speech, language and communication, if it is not diagnosed and intervened in the early period. Early diagnosis and early intervention programs are especially important for parents who want their children with hearing loss talk. Behavioral and electrophysiological tests are used in evaluating hearing in infants and children. Each of these tests has its own technical features.

The key factor for achieving a reliable test result is the selection of the appropriate test protocol. It is also important for the child to know the cognitive level and physical abilities of the child. Also, the cross-check principle is very important during this evaluation process (1).

It is very important to use a combination of test techniques in the evaluation of auditory functions in infants and children. As mentioned earlier, the use of a test-battery approach called the cross-check principle in determining the child's auditory performance is essential for correct diagnosis.

The crosscheck principle is described by Jerger and Hayes (1976) and is based on the determination of the child's auditory function by various behavioral and electrophysiological tests (2,3).

Assessing hearing in infants and young children requires a long time and cooperation.

Despite these limitations, responses from behavioral tests that are reliably performed in infants can be very useful both in detecting hearing conditions and in amplification.

Evaluating the peripheral hearing sensitivity that eliminates or confirms hearing loss as a cause of the problem of the infant and/or child, having knowledge about middle ear functions of the infant and/or child, assessment of auditory functions, if possible, using speech perception measures and observing and commenting on the auditory behavior of the infant and/or child are four main objectives of audiological assessment.

As a result of a comprehensive and successful pediatric audiological evaluation, an audiologist must have achieved the following results;

-The diagnosis of hearing loss

-Detection of presence or absence of auditory neuropathy

-Determination of the possibility of auditory processing or language impairment, if there are any

-The nature of the hearing based on behavioral and electrophysiological test results

-In due course, recommendations for comprehensive audiological and physical development and therapy-treatment

-Implementation of the monitoring, surveillance and hearing loss habilitation plan

-Family-oriented consulting and training services (4).

The American Speech-Language-Hearing Association (ASHA) and American Academy of Audiology (AAA) states that hearing loss is diagnosed in babies between 0-4 months, and behavioral tests for hearing instrument selection are not the preferred method of assessing hearing. There are four reasons for this. These include: a period of co-operation for the baby, a long test time and environment need, poor frequency information, and poor test-retest reliability (5,6).

Audiologic test protocols for children

0-6 months:

When babies are too young or have severe developmental deficits, it is recommended to use electrophysiological testing methods such as ABR and ASSR using frequency specific stimuli, primarily to estimate possible audiograms. Also, OAE and acoustic immunity measurements should be done in addition to ABR and ASSR. Patient's history, information provided by the family/caregiver, behavioral observation of the answers given by the baby to various sounds, developmental screening and functional audiovisual evaluations are also very important (1,4).

5-24 months:

In this age range, behavioral tests, i.e. Visual Reinforcement Audiometry, should first be performed. OAE and ABR are quite useful when behavioral audiometric tests are not reliable, when specific thresholds for the ear cannot be obtained, when results cannot be obtained from behavioral tests, or for auditory neuropathy.

25-60 months:

Visual Reinforcement Audiometry or Conditioned Play Audiometry and acoustic immunity measurements are usually considered sufficient. Speech perception tests should also be used in addition to developmental screening and functional auditory evaluations (1,5,6).

Behavioral Audiologic Tests

The information provided by behavioral tests on hearing measurement is very valuable. The main purpose of the behavioral tests is to determine hearing thresholds, especially for speech frequencies, for each ear and to assess speech perception at the supra-threshold level if possible. This information is crucial for the determination of amplification (hearing aid / cochlear implant), auditory habilitation and training strategies.

Appropriate behavioral procedures are directly related to the child's developmental, cognitive, and language levels and visual-motor development and skills (1).

There are three behavioral test techniques used in the assessment of hearing in infants and children. Each of these is suitable for children at different developmental levels.

Behavioral Observation Audiometry (BOA)

BOA can be used effectively in newborns and babies whose development is less than 6 months. It is a test used to determine the development of auditory skills in infants and is based on observing the behaviors shown by the infant in response to the sound provided. In some sources, it is stated that the term 'audiometry' is not used only for tests that measure hearing ability, that this test does not determine hearing thresholds and therefore it should be called 'Behavioral Observation'. This test method is not suitable for hearing screening, detecting hearing thresholds, selecting amplification technology, or device settings. In the test, the auditory stimulus behavioral response of the baby is observed and the results of the observation contribute to the evaluation of the development of auditory skills (1).

The observation should be carried out in a quiet room. Although autoscopic examination is not a prerequisite, it is useful to examine the outer ear deformities and abnormalities. If the baby is quiet and calm or slightly drowsy (REM) during the test, ensure that the baby sits on the baby seat or a pillow. If possible, it should not be on the lap of the mother or father. If the baby is on the parent's lap, their masking effect should be taken into account. Besides, when a voice prompt is given, it should also be prevented that the parent has any clue as to the presence of voice.

Complex acoustic stimulation (speech sound, speech noise, etc.) may be preferred at 60-90 dB HL, which is provided as an audible stimulus via an audiometer. The duration of the stimulation should be approximately 3-4 sec, because the duration of stimulation and response is longer in young babies than in older babies. It is possible for the stimulus to be given two or three times before the baby gets used to it (7).

The response of the baby's startle reflex to the auditory stimulus presented between 60-90 dB HL suggests that the peripheral auditory stimulation is normal. However, it is important to remember that the startle reflex is affected very quickly by physiological effects such as hunger and fatigue. Hence, the absence of a baby's startle reflex should not be considered as an immediate hearing loss, but should be assessed together with other observations and test results. Other behavioral changes the baby have shown in response to the stimulus should be stated as "present" or "absent", and should not be interpreted as a threshold or minimum response level. Infants may also respond to auditory stimuli, in such ways as increased and/or decreased sucking reflexes, respiratory changes (increased or decreased), moving or staying immobile, listening, blinking (1,8,9).

Visual Reinforcement Audiometry (VRA)

VRA is suitable for children with cognitive development between 5 months and 36 months. The test is a behavioral hearing test used to determine the frequency and ear-specific hearing sensitivity and the type of hearing loss using the conditioned response procedure. It is aimed that the hearing thresholds estimated at the minimum response level are similar to the actual perceptual thresholds.

It is recommended to use a speech stimulus in the Visual Reinforcement Audiometry and a warble tone or narrowband noise stimulus in the frequencies 500-1000-2000-4000 Hz. (1,5). However, in clinical practice, it is possible to arrange the frequencies tested according to the child's attention state and duration. For example, a child with a short attention span and a short test can get an idea of hearing thresholds at 250 (low) - 1000 (medium) and 4000 (high) Hz frequencies.

A quiet cabin should be preferred for testing. Earphones and silent cabin calibrations should also be made to comply with the international standards. Otoscopic examination is necessary for cleaning the external ear canal and for determining the size of the insert earpiece to be used during the test. During the test, it is preferred that the child sits on a baby chair or on the lap of the parent/caregiver. For the children sitting on the lap of the parents/ caregiver, the masking of these persons should be taken into account and the parents should be warned not to give the child any clue about the presence of the sound.

Visual Reinforcement In audiometry, most children spontaneously turn their heads within 2-3 seconds after the first sound stimulus without classical conditioning. Some children may require classic conditioning, especially in those with developmental disabilities. The expected response is that the child turns the head 90 degrees with the sound stimulus (1,10).

The thresholds or minimum response levels that are consistent with normal hearing sensitivity vary depending on the child's age.

Conditioned Play Audiometry

This behavioral test method is used to assess the hearing sensitivity of the ear and the frequency in developmentally children who are between 30 and 60 months and provides information on the type, degree, and configuration of the hearing loss. Before this test in the silent cabinet, both the quiet cabin and the earphone calibrations must be prepared according to international standards.

Pre-test otoscopic examination is also very important. During the conditioning phase, the child is taught the motor movement/behavior he/she should do when he/she hears the sound in the test. The child continues to experiment the behavior desired until he/she understands and learns it. Speech thresholds should also be obtained in the test, which is performed with specific tonal stimulation, usually at a frequency of 500-1000-2000 and 4000 Hz (1).

Speech Audiometry (SA)

SA is an important part of the clinical audiology test battery in the evaluation and monitoring of auditory functions in children. Pure sound assessment tests do not provide enough information about auditory function, although they provide information about the type and degree of hearing loss. The fact that the person is able to use hearing for speech perception is essential for language and speech production.

Speech perception testing basically assesses how the child hears speech (1,8). The main purpose of the speech audiometry in infants and young children is to determine the ability to recognize, discern and perceive speech as well as to support the reliability of pure sound thresholds. It can be used in children whose developmental age is over 6 months.

Result

Today, both behavioral and physiological tests should be applied in some cases and electrophysiological tests should be applied in some cases in order to make a complete audiological assessment and confirm the test results.

Knowing the cognitive age of the child is necessary for choosing the audiological test that is appropriate for the child. In some cases, however, the child's chronological age and cognitive skills may not be at the same level. For this, it is necessary to get detailed information about the child from the family before the test.

The choice of the test to be used in such a situation is quite easy, but the reliability of the results obtained from the test as well as the selection of the behavioral test which is suitable for the child otherwise is very controversial (1,4).

The Joint Committee on Infant Hearing suggests that babies should be diagnosed with hearing loss in up to 3 months and should be treated in up to 6 months in line with the 1-3-6 rule. However, at least one electrophysiological measurement is recommended for the prediction of hearing thresholds for infants and young children under 3 years of age with hearing aids and/or cochlear implants (11).

Keywords: audiologic assessment, children, hearing loss, test

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Chapter 22

NON-SURGICAL WOUND HEALING METHODS

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1. INTRODUCTION

Wound is the deterioration of tissue integrity due to physical and chemical reasons. Wounds are divided into three according to skin integrity, contamination with pathogenic microorganisms (PM) and time of formation (Figure 1). According to skin integrity, they are divided into open wounds and closed wounds. Abrasions, cuts, lacerations, punctures, bruises, and gunshot wounds are open wounds. Crush and explosion effects are closed wounds. They are divided into clean and infected wounds according to contamination with PM. A clean wound is a wound without PM, tissue loss and infection. Infected wounds are wounds in which pathogenic microorganisms are present (delayed wounds, rough wounds, dirty and deep wounds, etc.). According to the time of formation, wounds are divided into acute and chronic. Acute wounds are wounds that heal in the expected time under normal conditions. There is no factor preventing the healing of these wounds and the healing is continuous. Chronic wounds are wounds that do not heal in about three months. These wounds recur constantly (Arab, Orakçı, Erbilen, & Şahin, 1994).

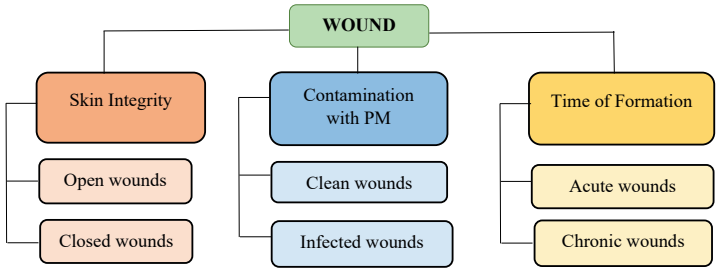

Figure 1. *Wound categories according to skin integrity, contamination with pathogenic microorganisms (PM), and time of formation.*

Wound care is a process that started in the history of humanity. The most important goal in the care of the wound is to restore tissue integrity as soon as possible, prevent infection and reduce pain (Daunton, Kothari, Smith, & Steele, 2012; Erdoğan, 2010; Gottrup & Leaper, 2004; Ovington, 2002). Some applications such as sterilization and disinfection have taken place in human life since the first ages of human history. There are various medical provisions in the inscriptions of the famous Babylonian King Hammurabi, who lived in Mesopotamia 2000 years before. By examining the papyri in Ancient Egypt, more information was obtained about the medical process of that period. According to the Ebers papyri, wounds were stuck to fresh meat with bandages on the first day. Hippocrates was the first to apply antisepsis by using tar (goudron) on wounds, he described primary and secondary wound healing. When Ambroise

Bare, who led the renaissance of surgery, found many wounded on the battlefield in Turin, he began to treat them with the burning hot elderberry oil. Nidai, one of the Turkish surgeons in the 16th century, wrote in his work that "it would be better if they rub the tar on the wounds on the hands and feet a few times and use the tormentil against the wounds" (Yalçın & Özkalp, 2005). Afterward, antiseptics used in the treatment of wounds were developed and made more useful.

Healing of all wounds is based on the same basic principles (Parsak, Sakman, & Çelik, 2007). The wound healing process begins immediately after the injury and follows a special sequence of three stages (Valero, Javierre, García-Aznar, Menzel, & Gómez-Benito, 2015; Yalçın & Özkalp, 2005). These stages are hemostasis, inflammation, proliferation, and maturation (Ud-Din & Bayat, 2014):

a) Hemostasis and Inflammation Phase (1-5 days): This phase begins approximately 10-15 minutes after injury (Parsak et al., 2007). The wound area fills with blood and intense bleeding begins (Arab et al., 1994). Substances such as histamine come out of the mast cells (a connective tissue cell containing granules called histamine and heparin) in the wound area, then vasodilation begins, and vascular permeability increases. As a result, leukocytes, monocytes, platelets, and plasma exit the vascular bed and migrate to the intermediate region (Parsak et al., 2007). As a result of this migration, a thrombocyte combined with blood forms a clot and stops bleeding (Wilkinson & Hardman, 2020; Yalçın & Özkalp, 2005). Platelets are the main contributors to the hemostasis stage and coagulation (Wilkinson & Hardman, 2020). Protein-structured growth factors are released from the alpha granules of the platelets in the clot. These factors initiate the healing process (Arab et al., 1994). In the absence of any of these factors, severe bleeding continues and wound healing is delayed. Fibrin formed during hemostasis is stored in the lymphatic system and causes a lymphatic blockage, and as a result, local signs of inflammation such as redness, edema, and fever are observed in the wound (Yalçın & Özkalp, 2005). Macrophages are formed from monocytes. While macrophages kill and phagocytize bacteria, they also remove dead tissue and leukocytes. Macrophages are most abundant in the wound 72 hours after injury. Macrophages provide the formation of the inflammation phase by secreting cytokines (Parsak et al., 2007). The inflammation phase is a complex process due to internal and external factors. Uncontrolled and excessive inflammation delays wound healing and promote tissue injury. Inadequate immune cell recruitment also delays healing (Wilkinson & Hardman, 2020). Meanwhile, the formation of new vessels begins. As the new vessel is formed, oxygenated blood reaches the wound area and the wound is fed better. This accelerates the development of granulation

tissue. Granulation tissue begins around the 5th day. The oxygenation and good nutrition of the tissue are essential for starting this stage (Parsak et al., 2007).

b) Proliferation Phase (5-14 days): The proliferation phase starts a few hours after the injury and lasts for weeks (Yalçın & Özkalp, 2005). This phase generally includes the formation of new epithelium and granulation tissue (Luo, Dai, Zhang, & Li, 2021). In this phase, the fluid accumulating in the wound area is replaced by new tissue and thus cell growth is achieved. Due to the epithelial tissue formed in the wound area, foreign substances are prevented from entering the wound, and the fluid and electrolyte balance of the wound is provided. Fibroblasts are attracted to the scar tissue by macrophages (Yalçın & Özkalp, 2005). Fibroblasts migrating to the wound area form collagen fibers. The collagen creates the tensile force of the wound. The collagen fibers combine with polysaccharides and mucoproteins to form a strong structure in the wound area, thus a strong bridge is established between the two walls of the wound (Parsak et al., 2007). New vascularization or capillary growth allows the formation of granulation tissue to fill the wound cavity. The color of the granulation tissue is dark pink due to the increase in the oxygen and nutrient vessels required for the growth of the newly formed scar tissue (Yalçın & Özkalp, 2005). When collagen production is sufficient, fibroblasts begin to disappear in the wound. This phase is completed when granulation occurs and epithelialization begins (Parsak et al., 2007).

c) Maturation Phase (after 14th day): It starts with the end of the proliferation phase (Arab et al., 1994). This phase lasts for years. In this phase, the collagen fibers are reshaped, and the type III (soft) collagen transforms into type I (hard) collagen. A certain collagen production-destruction balance is established within the collagen fibers that have accumulated and knitted the wound (Parsak et al., 2007). In this phase, the wound begins to mimic normal tissue. Fibroblasts and macrophages are also destroyed (Arab et al., 1994). The most important feature of this phase is the beginning of the reshaping function (Parsak et al., 2007) (Figure 2).

Remodeling consists of stages such as collagen replacement and fibrin withdrawal. While the skin can reach 80 percent of its former strength with remodeling, its properties such as elasticity and absorption capacity cannot return to normal. The new tissue formed after healing is weak and loose (Arab et al., 1994).

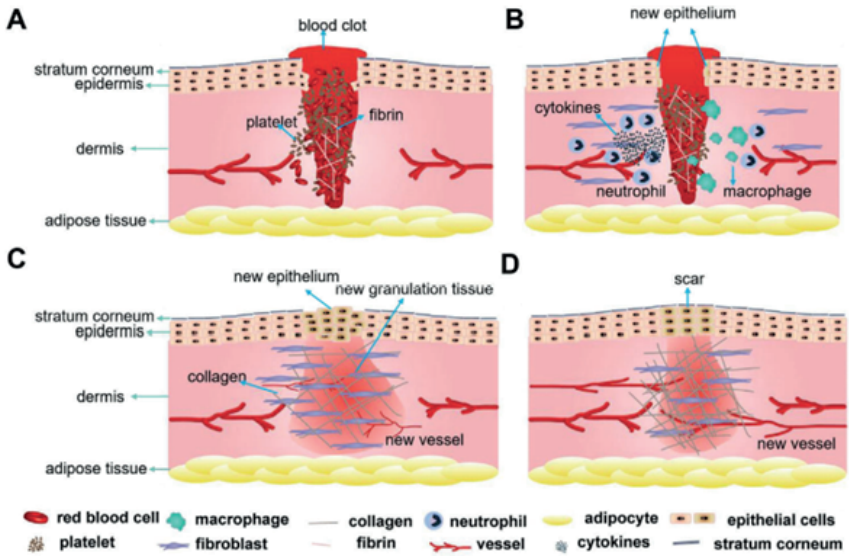

Figure 2. *In this phase, blood coagulation happens to maintain the wound. A) Blood clot occurs red blood cells, fibrin, and platelets. B) Inflammation phase. At this stage, macrophages, lymphocytes, and neutrophils that immigrate to the wound produce cytokines. C) Proliferation phase. At this stage, new epithelium and vessels are formed, while fibroblasts proliferate and migrate. D) Regeneration stage. This phase can cause scarring (Luo et al., 2021).*

Wounds that can complete their healing process in more than 42 days are called chronic wounds. A large number of people around the world suffer from chronic wounds. Chronic wounds amount to an average annual financial burden of 20-25 million dollars for the US healthcare system. The liability to treat these wounds is increasing due to the aging population, which puts a serious burden on healthcare costs (Kloth, 2014). It is extreme inflammation in the scar tissue that maintains the chronicity. At the same time, chronic wound infection can also be considered as the factor that creates chronicity. This increase in infection causes the wound to remain in a continuous cycle of infection, inflammation, and insufficient tissue repair. Interestingly, it has been observed that iron overload in the wound also delays the wound healing process (Luo et al., 2021). Factors such as diabetes, age, and vascular insufficiency are among the factors that will delay wound healing (Ud-Din & Bayat, 2014). In this review, surgical and non-surgical methods of chronic wounds will be explained in detail.

Surgical and non-surgical methods are available to treat chronic wounds. Surgical methods include negative pressure, debridement, and mechanical closure. All of these methods have complications; negative

pressure is bulky (takes up a lot of space) in diabetic ulcers and is unproven, debridement is potentially painful and does not help the biological wound healing process for diabetic ulcers, mechanical closure is limited by the size of wounds and does not help the wound healing process for diabetic ulcers. These methods are typically successful in treating chronic wounds but are very unlikely to work if the patient is elderly and/or diabetic (Braun, McGrath, & Downie, 2013).

The negative pressure method is a non-invasive method that accelerates the wound healing process by applying controlled pressure to acute/chronic wounds (El-Sabbagh, 2017; Huang, Leavitt, Bayer, & Orgill, 2014; Morykwas, Argenta, Shelton-Brown, & McGuirt, 1997; Webster et al., 2019). Fleischmann et al. first mentioned the application of negative pressure in the treatment of open and infected wounds (Fleischmann, Strecker, Bombelli, & Kinzl, 1993). In acute/chronic wounds, this application has become an increasingly common treatment method. The purpose of this method; is by keeping the wound clean, decreasing infection and edema, increasing blood flow to the wound area, and developing granulation tissue (A. Demir, Demirtaş, Çifci, Öztürk, & Karacalar, 2006). In this method, a sponge (vacuum) is placed on the wound bed and the fluid and other materials in the wound are drawn out with a hose. Infected tissue/fluid is removed, thus reducing the size of the wound but the patient is attached to the vacuum for a long time (Braun et al., 2013). In a study conducted by Demir et al. (A. Demir et al., 2006) , negative pressure therapy was applied to 50 patients with chronic wounds for an average of 12.4 days. The reduction in the size of the wound present in these patients averages 23 percent. This method has several effects that accelerate the process: 1) It increases the local blood flow rate. A study by Morykwas et al. demonstrated that a negative pressure of 125 mmHg increases blood flow rate fourfold, 2) Mechanical stress. Mechanical stress increases cellular proliferation and thus the rate of formation of granulation tissue increases, 3) It is the absorption of proteases that prevents healing (Morykwas et al., 1997). All these features ensure faster and healthier wound healing. The most common problems in negative pressure application are; pain, bleeding, tissue necrosis, infection, and inability to fully isolate the dressing and create an appropriate negative pressure (A. Demir et al., 2006).

Another surgical method used in wound healing is debridement (Bowers & Franco, 2020; Moya-López, Costela-Ruiz, García-Recio, Sherman, & De Luna-Bertos, 2020; Powers, Higham, Broussard, & Phillips, 2016; R. Zhao, Liang, Clarke, Jackson, & Xue, 2016). Debridement is the removal of the non-viable part from the scar tissue (Powers et al., 2016). Contaminated tissues found in a wound contain substances such as fibrinous, collagen, and elastin. All of these can be called debris or

necrotic tissue (Çelebi, n.d.). In chronic wounds, necrotic tissue inhibits the migration of keratinocytes onto the wound bed (Powers et al., 2016). This tissue creates a physical barrier against wound healing and causes an increase in the amounts of bacteria in the wound. The presence of such dead tissue may block normal wound healing. Therefore, removing the dead tissue on the surface of the wound accelerates wound healing (Çelebi, n.d.). Debridement can be performed surgically, automatically, enzymatically, biologically, and mechanically (Powers et al., 2016). In the debridement method, gauze is placed on the wound bed and salt is used to wet the dressing if necessary. It is allowed to dry, then the gauze is removed. The infected tissue is removed from the wound. In this way, new and living tissue remains in the wound so that the wound continues to close. Debridement can be painful for the patient and may require a temporary increase in wound size to be effective. This method may not be effective in diabetic patients (Braun et al., 2013).

Another surgical method is mechanical closure. If the wound is small enough, mechanical closure such as sutures, stapling and wound strips are applied. This method closes the wound to the external environment and reduces the wound size. However, mechanical closure is applied to limited wound sizes, leaves scarring, and may not be applied to wounds of diabetic patients (Braun et al., 2013).

Surgical methods are inadequate in chronic wounds such as diabetic wounds, pressure sores, and venous ulcers that heal very late. There are different wound healing methods that can be applied other than surgical procedures: electrical stimulation, ultrasound, and static magnetic field (Braun et al., 2013) (Figure 3). In this study, non-surgical methods are described in detail below.

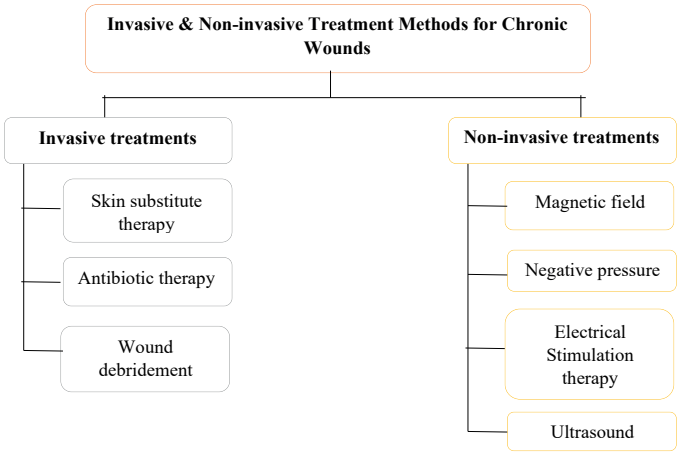

Figure 3. *Treatment methods of chronic wounds*

2. NON-SURGICAL METHODS OF WOUND HEALING

2.1. Electrical Stimulation Method

2.1.1. Injury Current

The definition of injury current was reported in 1843 by Du Bois-Reymond, who is known to measure the current of 1 mA in skin wounds (Balakatounis & Angoule, 2008; Sun, 2017). In later studies, it was confirmed that the electrical current in wounds was less than 1mA and the electric field surrounding the wound formed the definition of "injury current" (Balakatounis & Angoule, 2008). The endogenous electric field has emerged as a result of the bidirectional transport of polarized epithelial cells. Epithelial cells with Na+ and Cl- channels in the apical plasma membrane have Na+/K+ and ATPase in the basal plasma membrane. These ion channels with an asymmetrical distribution generate the current throughout the cell. Trans-epidermal potentials (TEP) are formed as a result of the directional transport of ions (Figure 4). Studies have shown that the wound flow occurs as a result of TEP short-circuited by epidermis damage (Luo et al., 2021; M. Zhao, 2009). It was observed that the injury current increased 2-3 mm around the wound and the electric field decreased from 140 mV/mm to 0 mV/mm (Balakatounis & Angoule, 2008). Compared to the surrounding healthy tissue, the wound site acts as a cathode (Luo et al., 2021). Current flowing through the wound path in the injured skin creates a lateral electric field. Therefore, injury current is thought to be important at the beginning of the repair of damaged tissue (Ud-Din & Bayat, 2014).

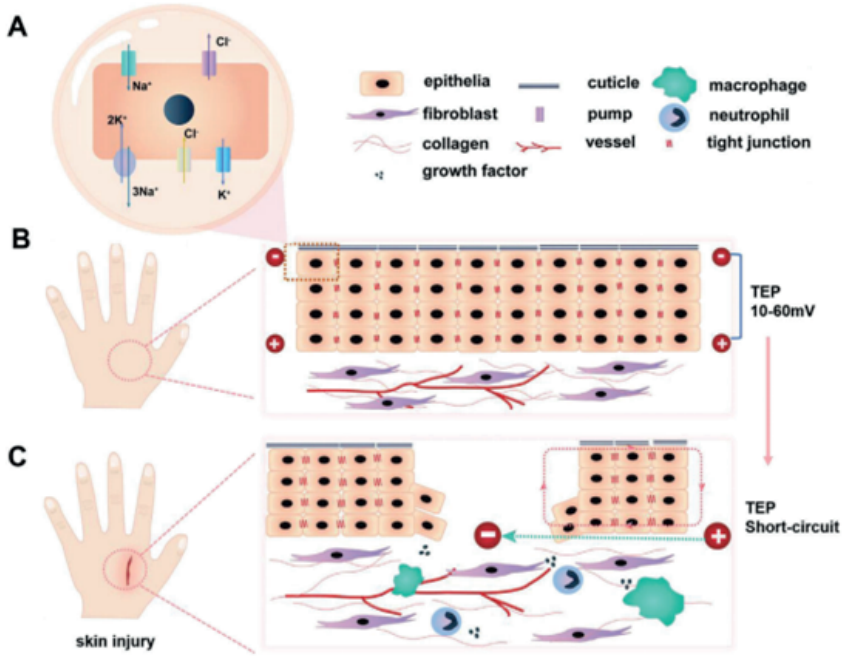

Figure 4. *Endogenous electric field and TEP generation in the wound. A) The K+ channel and Na+/K+-ATPase are in basal plasma and Na+ and Cl– channels are in apical plasma. B) TEP of healthy skin. TEP is formed as a result of the directional transport of ions. The electrical potential is higher in the lower part of the epithelium than in the upper part. C) Due to the short circuit of TEP, an electric field is generated in the skin wound. The positive pole of the electric field directed to the wound is at the edge of the wound and the negative pole is at the wound (Luo et al., 2021).*

2.1.2. Electrical Stimulation Method for Wound Healing

Electrical stimulation (ES) has the largest alternative to conventional wound treatments (Ashrafi, Alonso-Rasgado, Baguneid, & Bayat, 2016; Balakatounis & Angoule, 2008; Braun et al., 2013; Kloth, 2014; Luo et al., 2021; Mehmandoust FG, Torkaman G, Firoozabadi M, 2007; Sun, 2017; Thakral et al., 2013; Ud-Din & Bayat, 2014). ES is created by applying current with electrodes placed directly on the wound or close to the skin (Thakral et al., 2013).

Three types of current are commonly used in wound healing with ES as follows: direct current (DC), pulsed current (PC), and alternating current (AC) (Mehmandoust FG, Torkaman G, Firoozabadi M, 2007). DC current is a unidirectional ion flow for 1 s or longer (Figure 5). Depending on the polarity, the direction of the current flow is selected. If the DC

current flows for more than 1 s, it causes the secretion of substances such as NaOH, H2, HCl, and this effect may irritate the tissue (Kloth, 2014). Direct current mimics physiological endogenous current. Therefore, the cathode of the ES device is fixed to the center of the injured tissue, while the anode is fixed to the healthy skin around the wound (Luo et al., 2021). In wound care, low-intensity direct current (LIDC) (20-1000 □A) is used to prevent damage to healthy tissue (Ud-Din & Bayat, 2014). LIDC is a continuous monophasic waveform with a current of 1 mA or less (Luo et al., 2021). It has been observed that LIDC encourages wound healing by increasing the migration of fibroblasts and keratinocytes (Ud-Din & Bayat, 2014). The charged particles are arranged alternately when the bidirectional current of opposite polarity induces the wound. The electrodes of the ES device placed on healthy skin can highly prevent or even eliminate the thermal effect. Bidirectional flow is less invasive and has fewer adverse reactions compared to unidirectional flow (Luo et al., 2021).

Pulsed Current (PC) is a unidirectional or bidirectional flow of electrons or ions (Figure 6). The PC waveform, which is unidirectional/bidirectional flow of electrons/ions, is defined by its amplitude, frequency, and duration. This current is usually provided by electrodes placed on the wound or skin. PCs can have two waveforms: monophasic or biphasic. Monophasic pulsed currents (MPC) symbolize the movement of electrons or ions, returning to the zero line after a finite period of time. The duration of the monophasic pulse is always less than 1 ms (Kloth, 2014). The monophasic pulsed current usually consists of a high-voltage pulsed current (HVPC) or low-voltage pulsed current (LVPC) with a different electrode (treatment electrode) placed on the wound surface (Ashrafi et al., 2016). LVPC devices use continuous DC, monophasic and biphasic waveforms with longer duration and lower voltage (20–35 V). Using a single-phase and doubling current, HVPC has a short duration (less than 200 μs) and high voltage (150-500 V). HVPC is delivered by a device with negative and positive electrodes placed on the wound site or skin. This method applied with positive and negative polarity electrodes is used for wound healing and to reduce aches and edema (Ud-Din & Bayat, 2014). Bidirectional biphasic pulsed currents contain two phases (Kloth, 2014). Both electrodes used in treatments with biphasic pulsed current with an asymmetrical or symmetrical waveform are placed on the edge of the wound (Ashrafi et al., 2016).

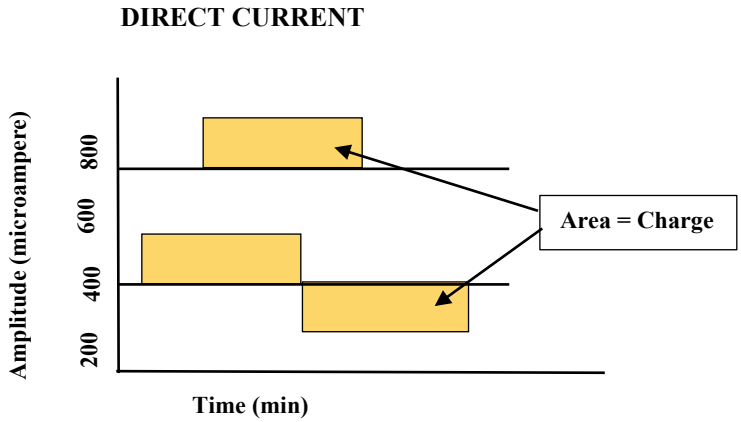

Figure 5. *Different types of DC waveforms (Kloth, 2014)*

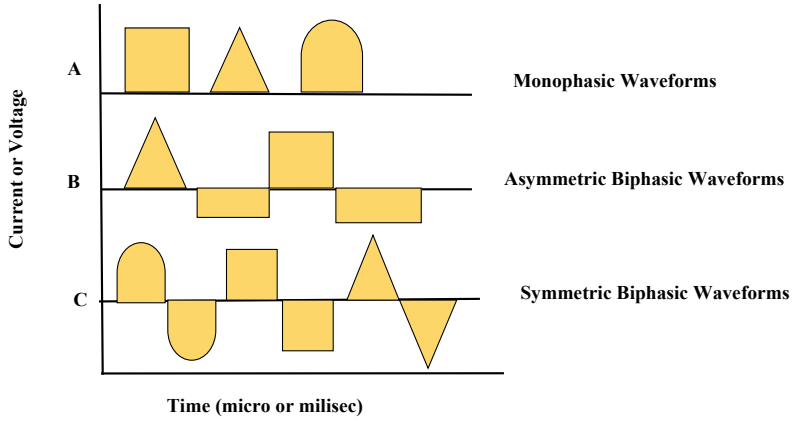

Figure 6. *Pulsed currents used for treatment are in monophasic or biphasic waveform. A) Monophasic B) Asymmetrical biphasic C) Symmetrical biphasic (Kloth, 2014).*

Transcutaneous Electrical Nerve Stimulation (TENS) is a well-known method to relieve acute and chronic pain and transmits a low frequency electrical current. In some studies (A. F. Cramp, Noble, Lowe, & Walsh, 2001; Kaada, E, & Eielsen, 1984; Nolan, Jr Hartsfield, Witters, & Wason, 1993; Simpson & Ward, 2004) it has been used to stimulate peripheral nerves and create physiological effects. Blood volume increased with low-frequency TENS compared to high-frequency TENS when applied at the same intensity (A. Cramp, Gilsenan, Lowe, & Walsh, 2000; Wikström,

Svedman, Svensson, & Tanweer, 1999). Frequency Rhythmic Electrical Modulation System (FREMS), another method used to heal wounds such as chronic leg ulcers, is a form of treatment that automatically changes the pulse, duration, and voltage. The Biofeedback ES device is used to heal scars on the skin and accelerate the healing process and is a low-density subcutaneous device. It monitors the standard electrical potential of the skin and monitors neural activity. The output of this device is 0.004 mA, 20-80 V, and the frequency is 60 Hz. The pulses of this device are lasting six hundredths of a second. It is thought that this device, which is claimed to eliminate the necessity of using continuous drugs, may also be useful in the treatment of abnormal healing wounds and skin scars (Ud-Din & Bayat, 2014).

2.1.3. Electrode Placement Method

Electrodes that deliver electrical energy to the scar tissue have two placement methods to deliver the current to the wound. In the first method, the treatment electrode is placed on the wound and the other electrode is placed on the intact skin. In the second method, two electrodes are placed on the upper part of the wound, one electrode (return electrode) on the intact skin, and a lead wire is bifurcated into them. The material structure of the electrodes consists of carbon, hydrogen, and polymer. The electrodes allow electric field to flow into the wound. They are chosen to be anode or cathode, hence, the cathode increases the mobility of epithelial, fibroblast, and keratinocyte cells; increases the motility of anode, macrophage, and neutrophil cells (Kloth, 2014).

According to studies conducted in different years, a significant reduction in wound size was observed when using negative polarity for the first 3 days and positive polarity for the remaining working days. The electrical stimulation method is an effective treatment method that causes a significant reduction in wound size, regardless of the applied polarity (Mehmandoust FG, Torkaman G, Firoozabadi M, 2007).

Table 1. *The literature review of skin wound healing by electrical stimulation methods.*

Reference	Type of current	Parameters	Model	Results
(Reich et al., 1991)	Unidirectional PC (Staodyn Vara/ Pulse Galvanic Stimulator)	Current: 35 *mA* / Time: 30 min (2 times per day) / Frequency: 128 *Hz*	Pigs (acute wound)	Inflammation phase shortened and mast cell count decreased
(Balay et al., 2004)	Unidirectional DC	Current: 300 *μA* / Time: 30 min per day	Rats (acute wound)	Inflammation phase shortened
(So et al., 2020)	bidirectional AC bidirectional BES (PC) Unidirectional DC	Voltage: 180 *mV* / Time:1 h (2 times per day) Voltage: 180 *mV* / Time: 1 h (2 times per day) Frequency: 1.688 *Hz* Voltage: 180 *mV* / Time: 1 h (2 times per day) /Frequency: 1.688 *Hz*	Fibroblasts (NIH-3T3)	The proliferation of collagen and fibroblasts promotion
(Burdge et al., 2009)	High-voltage pulsed (PC)	<140 *V*, 90-100 *μs*, 55.19 *Hz*	Human	Tissue healing was achieved
(Wirsing et al., 2013)	Wireless LIDC (DC)	1.5 *μA*	Human	Significantly accelerated healing
(A. Cramp et al., 2001)	TENS	High frequency: 110 *Hz*, 200 *μs* Low frequency: 4 *Hz*, 200 *μs*	Human	No difference in skin temperature and blood flow
(Ud-Din et al., 2013)	Biofeedback Electrical Stimulation	0.004 *mA*, 20–80 *V*, 60 *Hz*	Human	Improved scar symptoms
(Perry et al., 2010)	Biofeedback Electrical Stimulation	0.004 *mA*, 20–80 *V*, 60 *Hz*	Human	Improved scar symptoms
(Janković & Binić, 2008)	FREMS	300 *V*, 1000 *Hz*, 10–40 *μs*, 100–170 *μA*	Human	Accelerated ulcer healing and reduced pain wound area
(Goldman et al., 2004)	PC	100 pps, 360 *V*, -ve polarity	Human	Increased vasodilation and dermal capillary formation
(Franek et al., 2012)	PC	100 *V*, 100 *μs*, 100 *Hz*	Human	Improved healing rate

2.2. Wound Healing with Ultrasound

Researchers have proposed a different non-surgical wound healing method using ultrasound technology. Ultrasound is based on the mechanical vibrations conducted at a frequency above the limits of human hearing (20 kHz) (Ennis, Lee, Gellada, Corbiere, & Koh, 2016; Ennis, Lee, Plummer, & Meneses, 2011; Ennis, Valdes, Gainer, & Meneses, 2006). Therapeutic ultrasound (1-3 MHz), whose effect on wound care has been discovered recently, has been used for many years in physical therapy and sports medicine (Ennis et al., 2016, 2011, 2006). High frequency ultrasound (20-40 MHz) is used as diagnostic ultrasound for anatomical analysis of the skin (Ennis et al., 2006). Ultrasound has different effects on human tissues as cavitation, thermal effect, and mechanical effect. Cavitation, which enables the creation and use of micron-size bubbles

and fluids in the body, is an important mechanism of ultrasound. Microflow is created as the ultrasound beam moves liquids across acoustic boundaries with a mechanical pressure wave. Microflow produces a unidirectional fluid movement. This combination of microflow and cavitation (at the kHz level) provides mechanical energy that can alter cell membrane activity (Ennis et al., 2011, 2006).

The ultrasound transducer can drive by waves with different amplitude and different frequency, which is called as ultrasound dosage. These waves can be pulsed or continuous. The pulsed one has on/off cycles by changing the dosage of each element of the transducer. The second one has heating effect, however, if the intensity is chosen lower, there will be no thermal effect (Speed, 2001). The continuous ultrasonic waves can be generated by exciting the piezoelectric transducers with an external radio frequency source (Sahil Sharma, Mishra, Saini, & Dubey, 2020). The pulsed wave is tuned to transmit ultrasound pulses. Pulsing waves operating between 0.5 and 2 ms are separated by pauses of a few milliseconds (McCulloch, Kloth, & Feedar, 1995). These pauses in the pulsed wave shorten the energy received by the patient compared to the continuous wave. On wounds, a pulsed wave is used, since a non-thermal effect is required. There are some limits to the use of continuous ultrasound to be used for treatment: low intensity (<0.3 W/cm2), medium intensity (0.3-1.2 W/cm2), and high intensity (1.2-3.0 W/cm2). The lowest density should be preferred to create the desired effect. Low range is used for acute injuries, while chronic conditions use medium intensity. With each temperature increase, the density can be increased up to 0.5 W/cm2 depending on the increase. The lower intensity pulse wave can be used to avoid thermal effects (Michlovitz, 1996).

Ultrasound has physical and therapeutic effects and can harm the body if not used correctly (Hecox, Mehreteab, & Weisberg, 1994; McCulloch et al., 1995). Ultrasound has thermal and non-thermal effects. In thermal effects resulting from absorption, absorption depends on the size of the area to be treated, and as a result of this increasing thermal effect (Hecox et al., 1994), an increase in cell metabolism occurs (Davis & Ovington, 1993). Cavitation and acoustic flow are non-thermal effects (Hecox et al., 1994; McCulloch et al., 1995). Ultrasound can cause micron-sized bubbles to form in biological tissues such as blood and lymph, which can travel to different parts of the body. In response to pressure changes, ultrasound can cause bubbles to burst or vibrate (McCulloch et al., 1995). The researchers reveal that by ultrasonic cavitation the chemical effect can occur in the medium. If the medium is liquid, its temperature of it can increase. For this reason, the intensity of the excitation energy has an important role in the increment of temperature (S. Sharma, Yadav, & Dubey, 2020).

When performing therapeutic ultrasound, a matching medium should be used to reduce the effect of skin impedance. There are many oils, gels,

and degassed waters that allow the ultrasound waves to be applied more effectively to the related area. Recently, a therapy system was proposed to heal wounds using ultrasound technology, which is called MIST (Celleration, Inc., Eden Prairie, MN). With this non-contact system, the healing procedure is accelerating at a low frequency (Chang, Perry, & Cross, 2017). In MIST with saline, a transducer operating at 40 kHz is used at a distal displacement of 60-70 μs. Sterile saline creates a mist that acts as a conduit, allowing ultrasound energy to be transmitted to the wound bed without patient contact. The saline is sprayed on the wound surface vertically and horizontally for 4 minutes, with the device perpendicular to the wound bed. During treatment, the leading edge of the disposable treatment applicator is kept at a distance of 5-15 mm from the wound. Also, the distance between the front edge of the applicator and the emitting surface of the transducer is 10 mm, so the distance between the radiating surface of the transducer and the wound bed is 15-25 mm. Thus, at a distal substitution of 65 μs, the therapy intensity within the therapeutic range is 0.1 to 0.5 W/cm2 (Ennis et al., 2006).

Within vitro studies using ultrasound, the following effects have been observed: collagen production, increased macrophage response, increased angiogenesis, and increased nitric acid (Doan, Reher, Meghji, & Harris, 1999; Francis, 2001; Ito, Azuma, Ohta, & Komoriya, 2000; Maxwell et al., 1994; Reher, Harris, Whiteman, Hai, & Meghji, 2002; Young & Dyson, 1990b, 1990a). The effect of ultrasound on macrophages has been demonstrated, with evidence of an increase in cytokine production, leukocyte adhesion, and migration during the inflammatory phase (Doan et al., 1999; Ito et al., 2000; Maxwell et al., 1994; Young & Dyson, 1990a). As a result of ultrasound treatment, increased degranulation of mast cells (Fyfe & Chahl, 1984). increased collagen production with the growth of extracellular matrix (Doan et al., 1999), and morphological changes in fibroblasts were observed (Lai & Pittelkow, 2007).

Moreover, experimental studies using animals and humans were carried out with the MIST ultrasound device. MIST ultrasound used in a diabetic mouse model has been observed to increase angiogenesis and collagen accumulation (Thawer & Houghton, 2004). In another study by Ennis et al using MIST ultrasound (MIST Therapy; Celleration, Inc., Eden Prairie, Minn.), after 12 weeks of treatment, ulcers that had been faked healed by 40.7 percent, compared to 14.3 percent (p=0.0366) (Ennis et al., 2005). Ennis et al. in a study with the MIST therapy method, they found that the treatment was fast, painless, and clinically effective. In this study, the recovery rate was proven to be 69 percent (Ennis et al., 2006).

2.3. Wound Healing with Static Magnetic Field

Static magnetic field (SMF) is an alternative and non-invasive method apart from ES and ultrasound, which can produce therapeutic results (Lim, Cho, & Choung, 2009; Yun, Ahn, & Park, 2016; J. Zhao, Li, Deng, Yun, & Gong, 2017). Although the static magnetic field has less support in the scientific literature, magnets have been used to accelerate the healing process and reduce pain, especially in alternative medicine. Although there is not enough information, it is proposed that there are three effective mechanisms of the static magnetic field according to the relevant literature: 1) anti-inflammatory effect; 2) support for endothelial cell proliferation; 3) support for the formation of collagen (Henry, Concannon, & Yee, 2008). SMF is assumed as a strong stimulator of cell proliferation, migration, and differentiation, and has been observed to accelerate the differentiation of rat and human osteoblast-like cells in vitro (Yun et al., 2016).

In a research study, it was shown that SMF can affect the production of inflammatory cytokines released by macrophages and lymphocytes (Vergallo et al., 2013). SMF also has been approved by the US Food and Drug Administration (FDA) for applications in the treatment of pain and edema by modulating cell metabolism and proliferation (Zhang et al., 2016). Prolonged exposure to SMF can help control hypertension (Tasić, Djordjević, De Luka, Trbovich, & Japundžić-Žigon, 2017) and also plays a positive role in the treatment of osteoarthritis and nonunion fracture (Brown, Ling, Wan, & Pilla, 2002; Darendeliler, Darendeliler, & Sinclair, 1997; Zhang et al., 2016). In 2008, a study showed that a 220 mT static magnetic field increased the recovery rate in normal rats (Henry et al., 2008). In a recent study, the effects of an externally applied electromagnetic field, a 230 mT static magnetic field produced by a permanent NeFeB magnet. In another study, the effects of a static magnetic field on cutaneous wound healing in Streptozotocin (STZ)-induced diabetic rats were investigated (J. Zhao et al., 2017). In a study conducted in 2016, a pulsed electromagnetic field (PEMF) signal with a frequency of 75 Hz, a square waveform, and a magnetic field intensity of 1 mT was used. This signal was applied to rats for 1 hour with a Helmholtz coil. At the same time, pulsed radio frequency energy (PRFE) was applied at 27.12 MHz carrier frequency. It was observed that wound healing was faster in rats in which both treatments were applied compared to those that did not apply (Gümüşay et al., 2016). In a recent study, it was observed that moderate-intensity SMF (0.6 T) significantly improved wound healing in a mouse model of type 2 diabetes induced by a genetic mutation. Meanwhile, even SMF concentrations of less than 180-230 mT showed bene-

ficial effects on streptozotocin-induced diabetic wound healing in mice (Shang et al., 2019).

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Chapter 23

ALL ABOUT PLANTAR FASCIITIS - A NARRATIVE REVIEW

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Introduction

What is PF

PF is a common overuse injury that affects the tissue that runs along the bottom of the foot (Lopes, Hespanhol, Yeung, & Costa, 2012). However, when the plantar fascia is overused or strained, it can become inflamed and cause pain and discomfort. This condition, known as plantar fasciitis, is characterized by pain and stiffness in the heel and arch of the foot and is often worse in the morning or after periods of rest. In addition to the plantar fascia, other structures in the foot may also be involved in plantar fasciitis. These include the bones, muscles, tendons, and ligaments in the foot and ankle, which all work together to support the body and allow proper movement. The lifetime incidence is around 10%, constituting 11-15% of all foot pains (Crawford & Thomson, 2003). The incidence is relatively high in the 40-60 age group, and young runners and military personnel are the most common groups (Abate et al., 2009).

Anatomy of Plantar Fascia

The plantar fascia is a single, continuous robust, elastic, and thick band of connective tissue that runs along the bottom of the foot from the heel) medial tuberosity of calcaneus) to the base of the toes (metatarsal bones). It is composed of a dense network of collagen fibers that give it strength and elasticity. These fibers are arranged in a specific pattern to support the arch of the foot and help distribute forces evenly across the foot. The plantar fascia is attached to the heel bone by a small, fibrous area known as the plantar fascia insertional area. From there, it fans out along the bottom of the foot and attaches to the bones of the toes (Stecco et al., 2013).

The plantar fascia is a crucial part of the foot's anatomy and plays a key role in helping the foot to function properly. It helps to support the medial arch of the foot and provides a robust and springy structure that helps to propel the body forward when walking or running. The plantar fascia is also essential for absorbing shock and protecting the foot from injury.

Overall, a thorough understanding of the anatomy of the foot and the role of the plantar fascia is vital for properly diagnosing and treating plantar fasciitis.

- The plantar fascia is a thick band of connective tissue that runs along the bottom of the foot, from the heel bone to the base of the toes. It helps to support the arch of the foot and allows for proper movement during walking and running.

- The plantar fascia is attached to the heel bone (calcaneus) at one end and the base of the toes (metatarsals) at the other. It is anchored to the bones and muscles in the foot by a network of ligaments and tendons.

- When the plantar fascia is overused or strained, it can become inflamed and cause pain and discomfort. This condition, known as plantar fasciitis, is characterized by pain and stiffness in the heel and arch of the foot.

- In addition to the plantar fascia, other structures in the foot may also be involved in plantar fasciitis. These include the bones, muscles, tendons, and ligaments in the foot and ankle, which all work together to support the body and allow for proper movement.

- A thorough understanding of the anatomy of the foot and the role of the plantar fascia is vital for properly diagnosing and treating plantar fasciitis. This may involve imaging tests, such as X-rays or MRI scans, to assess the extent of the injury and guide treatment.

PF and the Windlass Mechanism

The windlass mechanism is a term used to describe how the plantar fascia and other structures in the foot work together to support the foot's arch and help the body move forward when walking or running. This mechanism is called the windlass because it works similarly to a pulley or winch, with the plantar fascia acting like a rope wound around the toes and heel. When the foot hits the ground, the plantar fascia is stretched and tightened, pulling on the heel and toes to help support the foot's arch. As the body moves forward, the toes are flexed and the plantar fascia is wound even tighter around the heel, providing an additional energy boost to propel the body forward. This process repeats with each step, supporting the arch of the foot and helping the body move forward efficiently and with minimal effort. In individuals with plantar fasciitis, the plantar fascia becomes inflamed and painful, which can interfere with the normal functioning of the windlass mechanism and lead to difficulty walking or standing for long periods. Treatment for PFtypically focuses on reducing inflammation and supporting the foot arch to restore normal function and relieve symptoms (Bolgla & Malone, 2004).

Pathophysiology of PF

The pathophysiology of PFis not well understood, but it is thought to involve a combination of mechanical and biochemical factors. Mechanical factors that may contribute to PFinclude overuse or strain on the plantar fascia, poor foot mechanics, and structural abnormalities of the feet (Gariani, Waibel, Viehöfer, & Uckay, 2020). For example, people with flat feet or high arches may be more susceptible to PFdue to increased

strain on the plantar fascia. Biochemical factors that may play a role in the development of PFinclude inflammation and changes in the composition of the plantar fascia (Wearing, Smeathers, Urry, Hennig, & Hills, 2006). Inflammation can cause the tissue to become stiff and painful and can also lead to the formation of small tears in the plantar fascia. These tears can cause further inflammation and pain, leading to a cycle of injury and pain. Overall, the pathophysiology of PFis complex and not fully understood. Further research is needed to understand the underlying mechanisms of this condition entirely.

Etiology of PF

Several risk factors may increase a person's likelihood of developing plantar fasciitis, including intrinsic and extrinsic factors (Lim, How, & Tan, 2016).

Intrinsic risk factors are related to the individual and may include age, weight (obesity), foot structure (pes planus), reduced ankle motion, prolonged standing, and previous foot injuries. People who are older, overweight, have flat feet or high arches, or have a history of foot injuries are more likely to develop PF (Scher, Belmont, & Owens, 2010). On the other hand, extrinsic risk factors are related to external factors such as the type of footwear a person wears and the types of activities they participate in. People who wear shoes that do not provide enough support for the arch of the foot or who engage in activities that place much stress on the feet, such as running or dancing, are more likely to develop PF (Scher et al., 2010). Other factors that may increase the risk of PF include standing for long periods on firm surfaces, having tight calf muscles, and having certain medical conditions such as diabetes or rheumatoid arthritis (Orchard, 2012).

This inflammation can be caused by several factors, including overuse or overstretching of the plantar fascia, obesity, and changes in walking or running gait (Sobhani, Dekker, Postema, & Dijkstra, 2013). Some people may be more prone to developing PF due to age, foot structure, or certain occupations that put extra strain on the feet. PF is often seen in people who engage in activities that strain the feet, such as running, dancing, or standing for long periods (Sobhani et al., 2013). It is also more common in people who are older, obese or have certain foot structures that make them more susceptible to the condition.

PF Diagnosis and Assessment

The physical examination is an integral part of the diagnostic process for plantar fasciitis. Patients typically describe a gradual onset of pain in the bottom of the heel that worsens when they take their first steps in the

morning or after rest (Buchbinder, 2004). The healthcare provider will inspect the feet for any signs of swelling, redness, or other abnormalities during the examination. They may also palpate (feel) the bottom of the feet, particularly the heel and arch, to assess for tenderness or other areas of pain. In addition to these general examination techniques, the healthcare provider may perform specific tests to assess the function of the plantar fascia and identify areas of discomfort or difficulty. These tests may include the following:

- The Thompson test, which involves pressing on the bottom of the foot to assess the function of the plantar fascia
- The single-leg heel rise test, which involves standing on one leg and raising the other heel off the ground to assess the strength and flexibility of the plantar fascia
- The single-leg calf raise test, which involves standing on one leg and raising the heel off the ground to assess the strength of the calf muscles

During these tests, the healthcare provider will look for signs of discomfort, difficulty, or pain in the heel or arch and any other abnormalities that may indicate plantar fasciitis. They will also assess the patient's overall range of motion and foot function to identify other potential contributing factors to their symptoms.

The diagnosis of PF is typically based on a person's symptoms, medical history, and a physical examination of the feet (Cole, Seto, & Gazewood, 2005). During the diagnostic process, the healthcare provider will ask about the location and nature of the pain, as well as any other symptoms the person may be experiencing. The provider will also ask about the person's medical history, including any previous injuries or conditions that may be contributing to the pain (Cole et al., 2005).

The provider may also ask about the person's overall health and any other medical conditions relevant to the assessment, such as obesity or diabetes. Next, the provider will perform a physical examination of the feet. This may involve checking the range of motion in the feet and ankles and testing for tenderness or swelling in the heels or arches. The provider may also ask the person to perform certain movements or exercises to assess their gait and overall foot function (Thong-On et al., 2019).

In some cases, imaging tests such as X-rays, ultrasonography and MRI scans may be ordered to confirm the diagnosis or rule out other potential causes of heel pain (Mohseni-Bandpei et al., 2014). These tests can provide detailed images of the feet and help the provider determine the extent of the injury and the best course of treatment.

Overall, the diagnostic process for PF is important for identifying the underlying cause of the pain and developing an effective treatment plan.

In addition, several tests may be used to diagnose plantar fasciitis, including:

1. Blood tests: In some cases, the provider may order blood tests to rule out other potential causes of heel pain, such as infection or inflammation.

2. Nerve function tests: If the provider suspects that a nerve disorder may be contributing to the pain, they may order nerve function tests to assess the function of the nerves in the feet.

Overall, the diagnostic process for PF may involve a combination of different tests to diagnose the condition and guide treatment accurately. The assessment process may take several visits to the healthcare provider, depending on the severity of the condition and the response to treatment. The provider will monitor the person's progress and make any necessary adjustments to the treatment plan.

Sports Branches and Plantar Fasciitis

PF is a common condition among athletes (Kaya, 1996). Athletes who engage in high-impact activities or have certain foot structures or gait abnormalities may be more susceptible to developing plantar fasciitis. It can affect anyone, but it is more common in certain groups, including athletes who engage in activities that stress the feet. Athletes particularly prone to PF include runners, dancers, and other individuals who engage in high-impact activities that put much stress on the feet. This is because these activities can lead to overuse and strain of the plantar fascia, which can cause the tissue to become inflamed and painful. Other athletes at risk of developing PFinclude basketball players, tennis players, and gymnasts.

In addition to the type of activity, other factors that may increase an athlete's risk of developing PF include the type of footwear they wear, the surface they are playing on, and their overall fitness level. For example, athletes who wear shoes that do not provide enough support for the foot arch or who play on hard or uneven surfaces may be more likely to develop plantar fasciitis. Similarly, athletes who are not in good physical condition or have tight calf muscles may also be at increased risk.

Footwear Types and PF

There may be an association between shoes and PF (Rajput & Abboud, 2004). Shoes not providing adequate support and cushioning can put extra strain on the plantar fascia, leading to inflammation and pain. Wearing shoes with good arch support and a cushioned sole can help to

reduce strain on the plantar fascia and prevent or treat plantar fasciitis. It is also important to replace shoes when they become worn or do not fit properly, as this can also contribute to the development of plantar fasciitis.

Training Loads and PF

There may be an association between training loads and PF (Rathleff et al., 2015). Training loads that are too high or increased too quickly can put extra strain on the plantar fascia, leading to inflammation and pain. It is crucial for athletes and individuals who engage in regular physical activity to increase their training loads gradually and to listen to their bodies to avoid overuse injuries, such as plantar fasciitis. Consult a sports physical therapist for more information on safely increasing training loads.

Firm Surface or Ground and PF

There may be an association between firm surfaces and plantar fasciitis. Walking or running on hard, firm surfaces can put extra strain on the plantar fascia, leading to inflammation and pain (Werner, Gell, Hartigan, Wiggerman, & Keyserling, 2010). Using softer surfaces, such as tracks or grass, can help to reduce strain on the plantar fascia and prevent or treat PF. It is also important to wear shoes that provide adequate support and cushioning to reduce strain on the plantar fascia.

Calcaneal Spurs and PF

There is a potential association between calcaneal spurs and plantar fasciitis, but the exact nature of this relationship needs to be better understood. Calcaneal spurs, also known as heel spurs, are bony outgrowths that can develop along the bottom of the heel bone (calcaneus). These spurs are typically visible on X-ray images and can cause pain and discomfort when they rub against other structures in the foot. This condition can cause pain and discomfort in the heel and arch of the foot and is often associated with overuse or strain of the plantar fascia. Some studies have suggested that calcaneal spurs may contribute to the development of PF (Johal & Milner, 2012). It is thought that the bony outgrowths may irritate and inflame the plantar fascia, leading to pain and discomfort. However, more research is needed to fully understand the relationship between these two conditions and determine the most effective treatments. If a spur is seen in the x-ray with plantar fasciitis, it is essential to recommend soft-soled shoes in the treatment.

Hamstring and PF

There is no known direct association between hamstring and plantar fasciitis. However, there may be indirect associations between the two (Bolívar, Munuera, & Padillo, 2013). For example, tightness in the ham-

string muscles can lead to changes in gait, which can put extra strain on the feet and potentially lead to PF (Cooney, Sanders, Concha, & Buczek, 2006). Additionally, people with certain foot structures or gait abnormalities may be more prone to both hamstring tightness and plantar fasciitis.

There may be a relationship between flexibility and PF. Tightness in the muscles and connective tissue of the foot and lower leg can cause extra strain on the plantar fascia, leading to inflammation and pain. Stretching and increasing flexibility in the muscles and connective tissue of the foot and lower leg may help to reduce strain on the plantar fascia and prevent or treat PF.

PF and calf muscles

There may be a relationship between calf muscles and PF (Bolívar et al., 2013). Tightness in the calf muscles can cause changes in gait and can affect ankle biomechanics, which can put extra strain on the plantar fascia and lead to inflammation and pain (Cheung, Zhang, & An, 2004). Stretching and strengthening the calf muscles may reduce strain on the plantar fascia and prevent or treat plantar fasciitis.

Biomechanical alignment problem and Plantar Fasciitis

The plantar fascia provides support and stability to the foot, and abnormalities in the alignment of the foot and lower leg can cause excessive strain on the plantar fascia, leading to inflammation and pain. Some common biomechanical abnormalities that may contribute to PF include overpronation (excessive inward rolling of the foot), over supination (the excessive outward rolling of the foot), and excessive flatfoot (lack of arch in the foot) (Pohl, Hamill, & Davis, 2009). These abnormalities can cause the foot to be less stable and put extra strain on the plantar fascia, leading to inflammation and pain (Pohl et al., 2009). Correcting these abnormalities with orthotics or other interventions can help to reduce strain on the plantar fascia and prevent or treat PF (Xu, Wang, Ma, Ren, & Jin, 2019).

Surgery and PF

Treatment for PF typically begins with non-surgical options such as rest, ice, orthotics, and physical therapy. If these conservative measures do not provide relief, a surgical procedure may be recommended to release the tight plantar fascia and reduce the inflammation.

Surgery is generally only recommended for PF if more conservative treatments have not effectively reduced pain and improved function. Several surgical procedures can be used to treat plantar fasciitis, but they all involve cutting or releasing the plantar fascia to reduce tension and strain on the tissue (Al-Boloushi, López-Royo, Arian, Gómez-Trullén, & Her-

rero, 2019). Surgery can effectively relieve pain and improve function in some people with plantar fasciitis, but it carries risks and potential complications, such as infection and nerve damage (Cutts, Obi, Pasapula, & Chan, 2012). It is important to carefully weigh the risks and benefits of surgery with your doctor before deciding if it is the right treatment for you.

Several surgical procedures may be used to treat plantar fasciitis, including:

- Plantar fascia release: In this procedure, a small incision is made in the fascia to release the tight tissue and reduce inflammation (Morton, Zimmerman, Lee, & Schaber, 2013).
- Endoscopic plantar fascia release: This is a minimally invasive procedure that uses a small camera to guide the release of the plantar fascia through small incisions in the foot (Malahias, Cantiller, Kadu, & Müller, 2020).
- Gastrocnemius recession: In this procedure, the gastrocnemius muscle (one of the muscles in the calf) is lengthened to relieve tension on the plantar fascia (Arshad, Aslam, Razzaq, & Bhatia, 2022).

The specific surgical procedure used will depend on the individual patient and the severity of their plantar fasciitis. It is important to discuss the potential risks and benefits of each procedure with a foot and ankle surgeon to determine the best course of treatment.

Physical Therapy and PF

The best treatment for PF will depend on the specific needs and goals of the individual. In most cases, treatment for PF involves a combination of rest, ice, heat modalities, strengthening intrinsic foot muscles, taping and stretching exercises (Huffer, Hing, Newton, & Clair, 2017). Resting the foot can help to reduce pain and inflammation in the plantar fascia. Applying ice to the affected area for 15-20 minutes several times a day can also help to reduce pain and inflammation. Stretching exercises can help to improve flexibility and strength in the muscles and connective tissue of the foot and lower leg, which can help to reduce strain on the plantar fascia and prevent PF from recurring. Mobilization, myofascial release, massage, or foam roller massage can also help patients (Rambhia, Athavale, Shyam, & Sancheti, 2018).

In addition to these conservative treatments, a doctor or physical therapist may recommend mechanical treatment (orthotics and taping), laser treatment, iontophoresis, or other treatments to help speed up the healing process (Buchbinder, 2004; Osborne & Allison, 2006).

In addition to these treatments, several self-care measures can help to reduce pain in PF. These include:

- Wearing shoes with good arch support and a cushioned sole (Wilk, Fisher, & Gutierrez, 2000)
- Avoiding activities that put much strain on the feet, such as running and jumping
- Gradually increasing training loads to avoid overuse injuries
- Using softer surfaces, such as tracks or grass, for activities such as running or walking (Werner et al., 2010)
- Applying ice to the affected area for 15 minutes several times a day to reduce pain and inflammation (Barrett & O'Malley, 1999)
- Stretching the muscles (calf and hamstring) and connective tissue of the foot and lower leg to improve flexibility and reduce strain on the plantar fascia.

Some examples of stretching exercises that may be helpful for PF include (Digiovanni et al., 2006):

- Towel stretches: Sit on a chair and place a towel around the ball of your foot. Pull the towel towards your body to stretch the plantar fascia and the foot and lower leg muscles. Hold the stretch for 30-60 seconds and repeat several times.
- Calf stretches: Stand facing a wall with your hands on the wall for support. Step back with one foot and bend your front knee. Keep your back leg straight and your heel on the ground. You should feel a stretch in the calf of your back leg. Hold the stretch for 30-60 seconds and repeat several times on each leg.
- Arch stretches: Sit on the edge of a chair and place a rolled-up towel under the arch of your foot. Gently press down on the towel to stretch the arch of your foot. Hold the stretch for 30-60 seconds and repeat several times on each foot.

It is important to consult with a physician or physical therapist to determine the most appropriate stretches for your specific needs. Stretching should be done slowly and gently and should not cause pain. It is essential to listen to your body and stop stretching if you feel any discomfort.

Stretching and PF

Stretching is an important part of treating and preventing plantar fasciitis. Stretching can help to reduce pain and inflammation in the plantar fascia by increasing flexibility and reducing strain on the tissue (Di-

giovanni et al., 2006). It can also help to improve overall function in the foot and lower leg, which can help to prevent PF from recurring.

Many different stretches can be used to treat plantar fasciitis, and the best stretches for you will depend on your specific needs and goals. It is important to consult with a physical therapist to determine the most appropriate stretches for your condition. In general, stretches that target the muscles and connective tissue of the foot and leg, such as the calf, hamstring muscles and the plantar fascia, are most effective for treating PF (Bolívar et al., 2013). Stretching should be done slowly and gently and should not cause pain. It is important to listen to your body and stop stretching if you feel any discomfort.

ESWT and PF

Extracorporeal shockwave therapy (ESWT) is a treatment that uses sound waves to stimulate healing in damaged tissue. ESWT has been used to treat various conditions, including plantar fasciitis. A meta-analysis showed that ESWT was more effective in chronic PFwhile reducing morning pain and moderate-intensity ESWT decreased pain and high intensity ESWT is effective in functionality (Dizon, Gonzalez-Suarez, Zamora, & Gambito, 2013).

Nonsteroidal Anti-inflammatory Drugs NSAİDS and PF

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a type of medication that can be used to reduce pain and inflammation. They are commonly used to treat a variety of conditions, including plantar fasciitis. While NSAIDs may be effective in reducing pain and inflammation in the short term, they do not address the underlying cause of PFand are not a long-term solution for the condition (Lim et al., 2016). In addition, long-term use of NSAIDs can have potential side effects, such as gastrointestinal bleeding and kidney damage.

Dry Needling and PF

Dry needling is a technique that involves the use of thin needles to stimulate specific points in the body. It is sometimes used to treat plantar fasciitis, which causes pain and inflammation in the plantar fascia, the band of tissue that runs along the bottom of the foot. A metaanalysis showed that dry needling positively efffect pain intensity and pain related disability insort and long term (Llurda-Almuzara et al., 2021).

Manipulation and PF

Manipulation is a technique that involves using the hands or an instrument to apply gentle force to joints or other parts of the body to improve its function and range of motion. It is sometimes used to treat plan-

tar fasciitis, which causes pain and inflammation in the plantar fascia, the band of tissue that runs along the bottom of the foot. In a study (Dimou, Brantingham, & Wood, 2004) manipulation can effectively reduce pain and improve function in people with plantar fasciitis.

Mobilization and PF

Mobilization is a technique that involves using the hands or an instrument to apply gentle force to joint or other parts of the body to improve its function and range of motion. Some studies have suggested that mid-foot and ankle mobilization can effectively reduce pain and improve function in people with PF (Celik, Kuş, & Sırma, 2016; Fraser, Corbett, Donner, & Hertel, 2018). Moreover soft tissue mobilization can effectively reduce pain and improve foot function (Looney, Srokose, Fernández-de-las-Peñas, & Cleland, 2011).

Orthoses and PF

Orthoses, also known as orthotics, are devices worn inside the shoe to support the foot and improve its function. They are often used to treat conditions that affect the foot and lower leg, including plantar fasciitis. Some studies have suggested that using orthoses can help reduce pain and improve function in people with PF (Anderson & Stanek, 2013; Landorf, Keenan, & Herbert, 2006). However, the evidence is insufficient to recommend orthoses as a first-line treatment for plantar fasciitis. More research is needed to fully understand the effectiveness of orthoses for this condition.

Night Splints and PF

Night splints are a device that is sometimes used to treat plantar fasciitis. Night splints are designed to hold the foot in a position that stretches the plantar fascia while you sleep, which can help to reduce pain and inflammation. Some studies have suggested that night splints can effectively reduce pain and improve foot function in people with PF (Schuitema, Greve, Postema, Dekker, & Hijmans, 2019). However, the evidence is not strong enough to highly recommend night splints as treatment for plantar fasciitis. More research is needed to fully understand the effectiveness of night splints for this condition.

Injections and PF

Corticosteroid injections are sometimes used to treat plantar fasciitis. Corticosteroid injections can help to reduce pain and inflammation in the plantar fascia by delivering a powerful anti-inflammatory medication directly to the affected area. Some studies have suggested that corticosteroid injections can be effective in reducing pain and improving function

in people with PF (Celik et al., 2016; Thomas et al., 2010). However, the evidence is not strong enough to recommend corticosteroid injections as a effective treatment for plantar fasciitis. Using both steroid therapy and plantar stretching can be effective in relieving pain, but it is important to use ultrasound monitoring in conjunction with steroid injections to minimize the risk of complications. (Tatli & Kapasi, 2009). More research is needed to entirely understand corticosteroid injections' effectiveness for this condition. In addition, corticosteroid injections can have potential side effects, such as skin thinning and infection, so it is important to consult with a physician or orthopedist to determine the most appropriate treatment for your specific needs.

Conclusion

Treatment for PF often involves a combination of rest, ice, and stretching exercises. Resting the foot can help to reduce pain and inflammation in the plantar fascia. Applying ice to the affected area for 15-20 minutes several times a day can also help to reduce pain and inflammation. Stretching exercises (calf and hamstring) can help to improve flexibility and strength in the muscles and connective tissue of the foot and lower leg, which can help to reduce strain on the plantar fascia and prevent PF from recurring. If there is no positive response from physical therapy and persistent painful condition persists, surgery may be attempted. In addition to these conservative treatments, a doctor or physical therapist may recommend theraptic agents (ultrasound, high intensity laser therapy) orthotics, physical therapy, or other treatments to help speed up the healing process. It is essential to consult with a doctor and physical therapist to determine the most appropriate treatment plan for your specific needs.

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Chapter 24

THE USE OF ZINC NANOPARTICLES IN POULTRY NUTRITION

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Introduction

Animal protein sources, which have an important place in human nutrition, have been supplied from animals such as cattle, sheep, goats, pigs and poultry for hundreds of years. Among these, especially meat and eggs obtained from poultry have a special importance in meeting the protein requirements of the growing human population. Considering that the human population will increase approximately 9 billion, and the need for food will increase two or three times in 2050, it has become necessary to use modern technologies in animal production systems (Selokar et al., 2020; Sekhon, 2014). The increasing interest in the use of nanotechnology in agriculture and animal husbandry in recent years can be cited as an example.

In general, nanotechnology is a new scientific approach involving studies on the control of a naometer-sized substance at atomic or molecular level. The most important characteristic feature of this technology is the manipulation of very small-scale particles (1-100 nm) known as nano-size (Shafi et al., 2020). The increasing interest in nanotechnology in the field of diagnosis, medicine and nutrition in the last decade has also increased the interest in the potential application of nanomaterials in poultry nutrition (Nabi et al., 2020). Although the bioavailability of organic minerals has been proven to be higher than inorganic forms in previous studies, for example, the application of organic zinc to animal diets has been limited due to its high cost (Zhao et al., 2014). It has been thought that the use of nanoparticles as feed additives, which differ from their common forms due to both their size and uniform physical properties, will create an advantage in terms of bioavailability, small dose rate and stable interactions with other components (Fesseha et al., 2020). In recent years, the majority of studies with poultry have been related to the use of nano-minerals, and studies have suggested that nano-minerals prevent intestinal mineral antagonism, reduce fecal excretion, and increase immunity and health (Gopi et al., 2017). Requirements for the common inorganic form of zinc commonly used in poultry nutrition defined by Nantional Research Council (1994) were 35-45 mg/kg diet for chickens, 40-70 mg/kg diet for turkey, 40 mg/kg diet for goose, and 60 mg/kg diet for ducks. According to the European Food Safety Authority (EFSA, 2014) report, Zn requirements/allowance and recommendations were 35-70 and 70-140 mg/kg diet for chicken, 40-120 and 75-150 mg/kg diet for turkey, 40-60 and 75-105 mg/kg diet for geese, 60-70 and 75-135 mg/kg diet for duck, respectively. This situation revealed how high Zn was added to the feed. In this context, the use of Zn nanoparticles in poultry feed may be an effective alternative within the scope of nanotechnology, which has attracted attention in recent years. In this chapter, it was aimed to provide

general information on the use of Zn nanoparticles in poultry nutrition and to touch on the results of studies on this subject in recent years.

Properties and mechanism of action of nanominerals, and bioavailability of zinc nanoparticles

The unit of measurement for nanoparticles is the nanometer (nm), which is equivalent to one-billionth of a meter. To make a biological comparison; a leukocyte is 10000 nm, a bacterium is 1000-10000 nm, virus is 75-100 nm, protein is 5-50 nm, and DNA is ~2 nm (Bhagat et al., 2015). Nano minerals have particle sizes ranging from 1-100 nm and have the ability to remain stable under high temperature and pressure (Stoimenov et al., 2002). Nanominerals can be easily absorbed from the digestive tract and utilized by the body, and therefore they can be effective even at low doses compared to their natural forms (Feng et al., 2009). Nanominerals absorbed through the villi epithelium in the digestive tract participate directly in the blood circulation, disperse to various organs and enter the cell with the process of transitosis. Nanoprticles entered the bloodstream can be captured by the liver and spleen, and while larger particles such as 40 nm are captured by the 50-100 nm Kupffer cells in the liver, smaller particles such as 2 nm are filtered by the kidneys. Their small size increases their uptake from the gastrointestinal tract, which increases bioavailability and reduces excretion (Singh et al., 2020). In addition, nanominerals interact better with other biologically active substances in vivo due to their large surface area (Zaboli et al., 2013).

Bioavailability, which defines the degree of absorbed part of a nutrient ingested by the animal in the usable form in metabolism (Ammerman et al., 1995), has an important place especially for trace minerals. There are several factors such as as the amount of mineral intake, chemical form, particle size, the presence of other nutrients that may interact with the mineral, inhibitors, and the physiological and pathological state of the oraganism, that affect the bioavailability of minerals (Çetinkaya, 1998). Nanoscale minerals are generally rapidly distributed from the bloodstream to tissues, and the reticuloendothelial system involving organs such as the spleen and liver, are target tissues for nanominerals (Hassan et al., 2020). In this context, the adsorption of nano-zinc can be different compared to common forms, which are known and traditionally used, such as zinc sulfate or zinc oxide. Indeed, Tsai et al. (2016) reported that Zn-sulfate or the common ZnO form entered the blood circulation as Zn+2 via Zn-binding protein, while ZnO nanoparticles entered the bloodstream directly in the form of ZnO. While this is a less destructive and faster process on the intestinal villi, it also creates a result that greatly improves the bioavailability of zinc (Wedekind and Lewis, 1992). In addition to some biomarkers (e.g. metallothioneine mRNA and protein expression level for zinc) used

to determine the bioavailability of minerals (Martinez et al., 2004), measuring the concentrations of these minerals in target organs (e.g. bone zinc concentration, plasma zinc and alkaline phosphatase concentration, hemoglobin concentration for iron, liver measurements for copper) are also frequently used methods (Jondreville and Revy, 2003). Łukasiewicz et al. (2020) reported that there was no significant difference in terms of breast muscle, liver Zn concentrations and the amout of excreted Zn between the group given standard ZnO and the groups given nano-Zn at a rate of 75% and 100% of this standard ZnO level, however, the femoral bone Zn concentration was significantly higher than control. In a study on broilers to investigate the effects of the supplementation of 50g/ton feed of ZnO, Zn-methionine chelate, nano-ZnO and methionine coated nano ZnO, respectively, no significant difference was found for serum, liver and spleen Zn concentrations on day 21 and 35 (Alkhtib et al., 2020). However, Cho et al. (2013) reported that oral administration of ZnO nanoparticles exhibited dose-dependent absorption, increased the Zn content in the liver, kidney, spleen and brain, decreased its amount in feces, and this was a result of the high absorption of ZnO nanoparticles. Recently, the addition of nano-ZnO synthesized from an endophytic fungus (Alternaria tenuissima) to broiler diets at a rate of 40 and 60 mg/kg diet significantly increased serum, breast, thigh and liver ZnO concentrations compared to the control group, and also serum, thigh and liver ZnO concentration was significantly higher in the group supplemented 60 mg/kg diet nano-ZnO compared to the other experimental group (Hatab et al., 2022). In an experiment carried out to investigate the effects of nano Zn application in broilers before and after hatching (El-Damrawy et al., 2019), 0, 60, 80 and 100µg/egg nano-Zn was injected, and the highest breast meat Zn concentration in chicks obtained from eggs injected 100 µg nano-Zn and in the group supplemented 30 mg/kg diyet nano-Zn. Tibia Zn concentrations also increased depend on the increase of in ovo nano-Zn supplementation. In a study on broilers fed diets supplemented nano-Zn at a rate of 25, 50, 75, and 100% of the inorganic Zn used in the basal ration, it was determined that the Zn retention in the animal body increased in parallel with the increase in the nano Zn ratio in the diet, and this increase was significantly higher than the control group, and it was also suggested that this might be a result of the entry of nanoform of the mineral to the animal body by direct penetration (Asheer et al., 2018). In a trial conducted by Abedini et al. (2018a) in layer hens, no significant difference was found between the groups fed the diet containing 80mg/kg ZnO and ZnO nanoparticles in terms of pancreatic Zn content, however, Zn content in tibiotarsus, liver and eggs in the group supplemented with ZnO nanoparticles were significantly higher and excreted Zn in feces were significantly lower than the group supplemented with conventional inorganic ZnO. In an experiment

carried out by Tsai et al. (2016), diets contained 40 mg/kg ZnO were given to the control group and 60 mg/kg each of ZnO, organic Zn (Zn-methionine) and nano Zn were given to the experimental groups in laying hens, and no significant difference was found between the control and the experimental group in terms of Zn retention. However, Zn retention in both experimental groups given organic-Zn and nano-Zn was determined significantly higher than the control and other experimental groups.

Although it has been reported that the excretion of Zn-nanoparticles is less than conventional inorganic Zn forms, green synthesis of nano-Zn have also been investigated against the possible environmental accumulation and pollution possibility of the nanoparticles produced by physical and chemical methods. It has been reported that plant-derived nanoparticles are lower cost, easier producible and more eco-friendly than those produced by physical and chemical nanosynthesis (Rahimi et al., 2022). ZnO nanoparticles can be synthesized by biosynthesis from various plant extracts such as Cassia auriculata, Aloe vera, Duranta erecta, Cinnamomum verum, Bauhinia tomentosa, Vitex trifolia, Moringa oleifera, Azadirachta indica, Artocarpus gomezianus and Olea europaea (Abdelbaky et al., 2022). In an experiment conducted by Dukare et al. (2021) to investigate the effects of inorganic ZnO, green nano-ZnO (obtained biologically from Catharanthus roseus plant through green synthesis) and market nano-ZnO, each Zn source was added into broiler diets as 40, 60 and 80 ppm, and it was determined that bone dimension, bone weight, bone total ash, Zn concentration in bone , muscle and liver increased in animals fed diet supplemented with 80 ppm green nano-ZnO and market nano-ZnO.

Effects of zinc nanoparticles on growth and yield performance

As in every living creature, minerals that are essential for normal growth and many metabolic processes also have an important place in poultry nutrition. As in other farm animals, factors such as growth rate, feed efficiency and carcass characteristics are the parameters used in the determination of flock performance in the poultry industry, and in addition to various factors affecting these parameters, it is an inevitable necessity that minerals that affect nutritional metabolism, growth, immunity and skin quality by participating in metabolic reactions should be balanced in the diet.

In addition to organic and traditional inorganic minerals, researches on the effects of nano-minerals on poultry performance have accelerated in recent years. Considering the studies, it is noteworthy that zinc nanoparticles are the most researched nano mineral regarding poultry performance. This is probably due to the fact that zinc, as the main component of numerous enzymes known as metalloenzymes, plays an important

role in all performance and physiological processes in poultry, including energy, nucleic acid and protein metabolism (Torres and Korver, 2018). However, it is also known that zinc is an essential mineral involved in the structure and function of more than three hundred enzymes related to metabolism (Saleh et al., 2018).

Addition of nano ZnO to drinking water at a rate of 0.1, 0.2, 0.4, 0.6 and 0.8 ppm/L for 42 days resulted in a significant increase in live body weight gain (LBWG) in broilers compared to the control group, without any significant difference in feed intake (FI) and feed conversion ratio (FCR) (Krishna et al., 2022). In a study in broilers, the addition of 10 mg/kg ZnO nanoparticles to the diet for 42 days resulted in a significant increase in LBWG and improvement in FCR without difference in FI (Mahmoud et al., 2021). Similarly, in broilers fed a balanced diet, live body weight (LBW) was significantly higher in the experimental group fed diet supplemented with the same amount of nano-Zn as the Zn-sulphate given to the control group, and this improvement was 4.6% higher than the control group (Sizova et al., 2021). In a study carried out by Łukasiewicz et al. (2020) to investigate the effect of replacement of standard ZnO used in broiler diets with ZnO nanoparticles, the control group given 55mg/kg diet standard ZnO and the experimental groups given 25%, 50%, 75% and 100% of this zinc ratio as ZnO nanoparticles were compared and it was determined that only the group fed diet containing 100% ZnO nanoparticles had significantly higher LBW than the control group on day 42nd day of experiment, except for the 0th, 14th and 35th days of the experiment. Azza Hafez et al. (2017) reported that the use of ZnO nanoparticles at a ratio of 40 and 80 mg/kg diet in broilers significantly increased LBWG and feed efficiency without causing a change in feed consumption. El-Katcha et al. (2017) found that 60, 45 and 30 ppm Zn nanoparticle supplementation in broilers improved growth performance, while lower doses (15 ppm) caused a decrease in performance and feed efficiency parameters. On the other hand, the study carried out by Zhao et al. (2014), in which reported that Zn nanoparticles produced positive results in terms of performance in broilers even at low doses when compared with traditional ZnO, is remarkable. These researchers determined in their study that 20, 60 and 100 ppm nano ZnO in broilers created a significant increase in LBWG and feed efficiency compared to the control group supplemented with 60 ppm normal ZnO. While Ahmadi (2013) reported that the use of 40 mg/kg diet ZnO nanoparticles in broiler diets significantly increased feed efficiency and slaughter weight compared to the control group, Fathi et al. (2016) observed in their study, in which they used 0, 10, 20 and 40 ppm ZnO nanoparticles in broiler diets, that the best result in terms of LBWG and FCR was obtained from the group supplemented 20 ppm

compared to other levels. As a result of the experiment in broilers conducted by Alkhtib et al. (2020), the use of each nano-ZnO and methionine coated nano-ZnO (produced by a novel method) at a rate of 50g/ton diet significantly increased LBWG and FI compared to the control without creating a difference in FCR.

It has been also reported that zinc plays an essential role in insulin formation by participating in the structure of enzymes that play a role in insulin formation (McDowell, 2003). In this context, it was thought that Zn could have a positive effect on insulin like growth factor-I (IGF-I), which is one of the important marker genes in the development of growth rate in poultry (Junjing et al., 2018), and indeed, Ibrahim et al. (2017) showed that the addition of organic or nano-Zn to the diet in broilers significantly increased the expression of the messenger RNA gene of IGF-I, and even the addition of nano-Zn tended to be more effective in this. In a recent study conducted by Alian et al. (2022) on broilers, final BW, cumulative LBWG, cumulative FI, FCR, feed efficiency, performance index, European efficiency index, protein efficiency ratio (in grower and finisher phase) values were also found significantly higher in the group supplemented 40 mg/kg diet nano-ZnO than the group supplemented with normal ZnO. In this sudy, it was also revealed that nano-ZnO linearly elevated mRNA expression of IGF-I.

In addition to studies showing the positive effects of Zn nanoparticles on poultry performance, there are also studies reporting that there is no significant effect. Eskandani et al. (2021) found no significant difference for average daily gain (ADG), average daily feed intake (ADFI) and FCR between the control group supplemented 70 mg of Zn-sulphate, and the experimental groups supplemeted 30, 50, 70 and 90 mg of Zn nanoparticles per kg of diet, during the experiment. Similarly, Otowski et al. (2021) reported that no significant difference was found in turkeys in terms of BW, ADFI and FCR between conventional ZnO used at a rate of 10, 50 and 100 mg/kg diet and ZnO nanoparticles used at the same rate. Also, in a trial conducted in breeding quails with the same levels of the same zinc sources, it was observed that the addition of nano ZnO to the diet had no effect on BW, feed consumption and egg production (Tatlı et al., 2019).

Heat stress is an important environmental factor affecting poultry performance. Heat stress leads to pathological changes that trigger intestinal inflammation and destruction in poultry, reduces the absorptive surface area in the digestive tract and increases the susceptibility to infectious diseases by disrupting the intestinal barrier, thereby, indirectly affects performance negatively (Ebrahimi et al., 2015). A recent study has shed light on the fact that Zn nanoparticles can be an effective weapon in overcoming heat stress. In this study carried out by Abdel-Wareth

et al. (2022) in broilers exposed to heat stress at 37.8, 35.8 and 29.9 °C between days 1-10, 11-21 and 22-42, respectively, the administration of 20, 40 and 60 mg/kg ZnO nanoparticles to the diet resulted in significant improvement in LBW, LBWG and FCR compared to the control group. In addition, the digestibility of crude protein, crude fiber and ether extract nutrients also increased significantly in these experimental groups compared to the control group.

In recent years, studies have also been carried out on the effects of in ovo injection of Zn nanoparticles on embryo development, survival, hatchability and the growth performance of these hatched offspring. In ovo injection of 50, 75 and 100 ppm nano ZnO on the first day of incubation in fertile eggs decreased early embryo mortality, and significantly increased hatchability of eggs and the growth rates of the hatchlings in the grower phase than control without any change in feed consumption (Biria et al. al., 2020). In contrast, in an experiment conducted by Palouj et al. (2021), injection of 20 µL of saline solution containing 0.5 and 0.6 mg nano-ZnO in 50 mL into fertile broiler eggs significantly decreased hatchability and weight of hatched chicks, and embryonic mortality significantly increased in early (≤7 d) and late period (17-21 d). Also, no significant difference was found on day 42 between the control and experimental groups in terms of LBW and FCR of broilers obtained from these eggs. Likewise, Hamza et al. (2022) compared the effects of injection of 25, 50 and 75 µg of inorganic Zn-sulphate per egg in fertile broiler eggs and injection of the same amount of ZnO nanoparticles, and no significant difference was found between administrations in terms of hatachability percent, embrionic mortality, LBW and LBW percent of chicks. In ovo supplementation of 20, 40, 60 and 80 µg nano-zinc per egg on the 18th day of incubation in fertile broiler eggs was resulted in no significant difference between experimental groups and control for egg weight, hatch weight of chicks, ratio of chick weight to egg weight and hatchability percent (Joshua et al., 2016). Similarly, in the first of trials by Jose et al. (2018), in ovo application of 0.25 and 0.5 mg nano zinc per egg in 18-day-old fertile broiler eggs failed and the hatchability percentage was approximately 92% in the control group and 0% in the nano zinc groups. In the second experiment, 0.04 and 0.08 mg nano zinc per egg were applied and it was determined that the hatchability rate was 92% in the control group and approximately 81 and 83% in the nano-Zn groups, respectively. Also, in both trials, nano-Zn application did not make a significant difference in egg and chick weights.

As mentioned before, the negative effects of heat stress can also be seen on embryos. In cells exposed to heat stress, protein synthesis is inhibited as a result of changes in phosphorylation in many components of

the translational process, which leads to an increase in mortality and a rapid decrease in final BW (Syafwan et al., 2011). In a study conducted with the idea that in ovo mineral application can overcome these negativities in arid and semi-arid regions where heat stress is common, Hassan (2018) reported that in ovo application of 15 ppm nano Zn per egg in semi-arid conditions where indoor temperature was 35.7 °C and humidity was 24.2% did not make a significant difference compared to the control group in terms of egg weight, hatch weight of chicks, rate of chicks weight to egg weight and percentage of hatchability, however, LBW on day 35 and LBWG during the study were significantly higher than the control group in broilers obtained from these eggs.

In addition to the growth performance of the animals in the poultry industry, criteria such as egg yield and quality, organ weights, carcass characteristics and meat quality are also important parameters evaluated within the yield performance. In addition to obtaining the egg at the desired level, which is of great importance in meeting the protein requirement for healthy nutrition, the shell quality of the obtained egg has a special importance in preventing economic losses that may occur in this regard. It has been reported that the loss of eggs which cannot be offered for sale due to shell fractures varies between 6-20% (Atik and Ceylan, 2009). Minerals such as Zn, Cu, Mn can affect the mechanical properties of the eggshell through calcite crystal formation and its effect on the crystallographic structure of the eggshell (Mabe et al., 2003). Among these minerals, especially Zn plays an essential role in shell formation, since it is the cofactor of the carbonic anhydrase enzyme, which takes part in the formation of bicarbonate ions from water and carbon dioxide in egg shell formation (Atik and Ceylan, 2009). Considering that zinc is necessary for the functioning of this enzyme in the synthesis of bicarbonate, which is necessary for the formation of eggshell (calcium carbonate-CaCO3), it is possible that Zn nanoparticles may be effective in reducing egg fractures due to thinning of eggshell with age. Although the effects of Zn nanoparticles on eggshell quality were clearer, reports on egg production differed. The addition of 80 mg/kg ZnO-nanoparticle to the diet of laying hens did not make any significant difference in terms of egg production percentage compared to the normal ZnO supplemented group, except for the numerical increase, however, egg mass (g/hen per day), shell thickness. (mm), shell strength (kg/cm2) were significantly higher (Abedini et al., 2018a). Similarly, in a dose-dependent trial in layer hens by the same researchers (Abedini et al., 2018b), 40, 80 and 120 mg/kg ZnO nanoparticles were added to diet and the highest egg production was in the group given 80 mg/kg ZnO nanoparticles, however, egg mass, shell thickness and shell strength were significantly higher in the groups given 40 and 80 mg/kg

ZnO nanoparticles than the group given 120 mg/kg. In contrast, Tsai et al. (2016) found no significant difference in egg production, egg weight, eggshell strength and egshell weight between the group supplemented 60 mg/kg nano Zn and the group supplemented 40 and 60 mg/kg normal ZnO in aged laying hens (68 weeks old). They also reported that the eggshell thickness of the group given nano-ZnO was significantly higher than the group given normal ZnO. In a recent trial with laying hens, the addition of 20, 40 and 60 mg/kg nano-ZnO to the diet significantly increased egg weight, egg mass, egg production percentage, the Haugh unit, shell thickness and shell percentage, but there were no difference for the percentage of albumin and yolk (Fawaz et al., 2019). On the other hand, Javadifar et al. (2021) reported that the use of 100 and 200 mg ZnO nanoparticles per kg diet in laying hens did not have a significant effect on egg production and weight, however, haugh unit, eggshell stability, relative weight of eggshell, and specific gravity increased significantly compared to the control group.

In laying hens, molting has been practiced for many years in order to prolong the egg production of old hens. Instead of inhumane methods that stress the herd, such as deprivation of feed, starvation, even cutting off the water and reducing the time and intensity of lighting, which will cause the animals to molt, researchers have focused on the more humane alternative ways such as manipulations on the amount of minerals (e.g. Na, Ca, I, Zn) and the use of organic compounds to trigger molting within the scope of animal welfare and rights (Petek and Alpay, 2008; Meija et al., 2010). Recently, positive results were obtained in two studies carried out to investigate the effect of nano-ZnO as a trigger for forced molting in laying hens. The addition of 10, 15 and 20 g/kg nano-ZnO to the diet showed an inducer effect on molting, and the best result was obtained in the group given 20 g/kg. Also, in this study, the use of high levels of nano-ZnO significantly increased the egg production from the 2nd week following the end of molting to the end of the experiment compared to the control group (Al-Mosawy and Al-Hassani, 2022a). In another similar trial conducted by the same researchers, the use of 20 and 25 g/kg nano ZnO in the diet achieved the same success in triggering forced molting, and the egg production was significantly higher than the control group from the 3rd week of the end of the molting until the end of the experiment (Al-Mosawy and Al-Hassani, 2022b).

In addition to the growth performance of animals in the poultry industry, carcass and meat quality are also the important criteria evaluating within the scope of yield performance. The carcass yield, which increases in proportion to the live weight of the animal, decreases as the animal approaches its mature weight, depending on the amount of fat that increases

and therefore the amount of fat discarded during slaughter. This situation creates a loss in carcass yield and leads to a loss of marketable meat before offering for sale (Ceylan and Kutlu, 2022). Nutrition is an important factor among the factors affecting carcass characteristics and meat quality such as gender, age, heredity, accommodation, climate, physical and chemical properties of meat. Therefore, it is seen that parameters related to carcass characteristics and meat quality are also among the effects evaluated such as growth performance, immune system, antioxidant activity.. etc. in nutritional studies related to minerals. Various parameters such as water holding capacity, shear force, drip loss, cooking loss, pH, shelf life, collagen content, protein solubility, cohesiveness, fat binding capacity are used in the evaluation of meat quality (Allen et al., 1998). In addition to these, appearance and texture are also important parameters, and especially appearance (lightness, redness, yellowness..etc) is the most important quality parameter that affects the choice and satisfaction of the product by consumers (Yücesoy and Kaya, 2022). Meat quality parameters can be affected by both animal-related conditions and the relationship between parameters. For example; it has been reported that the high growth rate of the animal increases the lighness (L*) index of the meat (Fletcher, 1999), the increase in the fat content in the muscle increases the L* and yellowness (b*) index (Chartrin et al., 2006), the increase in the antioxidant status increases the redness (a*) index (D'Agata et al., 2009), the increase in pH in meat reduces the shelf life of meat (Allen et al., 1997), the decrease in pH increases the L* index (Berri et al., 2001), the sudden decrease in pH can lead to a decrease in water holding capacity (WHC) (Qiao et al., 2001). Considering that skin damage, which is frequently encountered in poultry farming, is closely related to the collagen content of the skin and that zinc plays an important role in collagen synthesis (Leeson and Summers, 2005) and that skin damage during production leads to extra moisture in the cooling tanks of the carcasses, resulting in a decrease in meat quality and shelf life (Salim et al., 2012), the importance of zinc in this context can be understood once again. Also, since zinc is involved in the structure of the superoxide dismutase (SOD) enzyme, which is effective in stabilizing structural membranes and protecting cells against lipid peroxidation (Maunier et al., 2005), it has been seen that many studies have been carried out on inorganic and organic zinc, with the thought that it can increase the stability and extend the shelf life of meat with its antioxidant effect. In this context, studies on Zn nanoparticles have been carried out in recent years, which also deal with carcass characteristics and meat quality in poultry. In a trial conducted in turkeys (Otowski et al., 2021), the use of conventional ZnO at a rate of 10, 50 and 100 mg/kg diet and ZnO nanoparticles at the same rate were compared and no significant difference was determined in terms of carcass yield, the percentage

share of breast muscles, drumstick muscles and intestinal fat in LBW. In terms of tigh muscle percentage, there was no difference between the group supplemented 50 mg/kg diet nano-ZnO and the group supplemeted 100 mg/kg conventional ZnO, but it was significantly higher than the other experimental groups. Also, there was no significant difference between the groups in terms of breast L* and b* values, but the highest value in terms of a* was determined in the groups supplemented highest ZnO regardless of the ZnO source. In a broiler experiment performed by Krishna et al. (2022), nano-ZnO was added to the drinking water at a rate of 0.1, 0.2, 0.4, 0.6 and 0.8 ppm/L for 42 days and no significant difference was determined in ready to cook yield, percent weight of heart, gizzard, giblet and abdominal fat as a percentage of LBW, however, the liver weight increased significantly depending on the increase in the nano-ZnO level, and the breast yield in the other experimental groups was significantly higher than the control group, except for the group given 0.8 ppm nano ZnO. Similarly, Hatab et al. (2022) reported that the addition of 40 and 60 mg/kg nano-ZnO to the diet in broilers caused a significant increase in carcass yield compared to the control group, and that the partial liver weight was also higher than the control group, but this increase was statistically significant only in the group given 60 mg/kg diet nano-ZnO. In a study on heat-stressed broilers, Abdel-Wareth et al. (2022) reported that the addition of 20, 40, and 60 mg/kg ZnO nanoparticles to the diet caused a dose-dependent linear increase in dressing percent, and this increase was significantly higher in the experimental groups supplemented nano-ZnO compared to the control group. They also reported that abdominal fat percent in the experimental groups was significantly lower than the control group, however, there was no significant difference in terms of liver, spleen, gizzard and heart percents. The addition of 30, 60, 90 and 120 mg ZnO nanoparticles to the basal diet containing 36.29 mg/kg inorganic ZnO at the starter stage in broilers increased significantly the dressing weight (with liver and heart) and net carcass weight (without liver and heart) of the groups given 60 and 90 mg/kg nano-ZnO compared to the other groups, but there was no significant difference between the groups in terms of breast weight, thigh weight, wing weight and abdominal fat percentages (Khah et al., 2015). In this study, it was also reported that the highest breast and thigh dry matter and crude protein percentages in terms of carcass quality parameters were determined in the groups supplemeted 60 and 90 mg/kg nano-ZnO, but no significant difference was determined between the groups in terms of ether extract. In a recent study carried out to investigate the effects of different Zn sources on broilers, the breast meat pH value was significantly lower, the L* value was higher, and the a* value was lower in the group given 40 mg/kg diet nano-ZnO compared to the control and the group given inorganik ZnO (Alian et al, 2022). Es-

kandani et al. (2021) reported that there were no singnificant differences for carcass traits between groups fed diet supplemented with 30, 50, 70, 90 mg Zn from nano-Zn (bonza Zn metabolism optimizer synthesized by using nanochelating technology) and control group fed diet supplemented 70 mg Zn from Zn-sulphate. Also, it was reported in this study that breast meat L* and a* color index were significantly higher, b* index drip loss and shear force were similar, meat pH value was significantly lower in groups supplemented nano-Zn compared to control group, and they recommended by taking into consideration other parameters (growth performance, humoral immunity, Zn and malondialdehyde concentrations in breast meat) that the diet supplemented with 30 mg Zn from nano-Zn should be used.

Effects of zinc nanoparticles on the immune system

It is known that zinc deficiency in animals causes weak immunity and creates susceptibility to many diseases. It has been reported that the addition of zinc to the diet, which has an important effect on the development of the immune system in poultry, improves antibody production (Cordoso et al., 2007). In broilers, the lymphoid tissue is divided into two groups, the central lymphoid containing the thymus and bursa, and the peripheral lymphoid containing the spleen, and these two lymphoid tissues are associated with the intestinal mucosa (Akter et al., 2006). Zn is a mineral that plays an important role in the maintenance of the structure and functionality of the lymphoid tissues and organs, which function in the protection of the animal against pathogens, and therefore in the development of the immune system (Kidd et al., 1996). It has been reported that zinc, which plays an essential cofactor in the formation of thymulin, which modulates cytokine release and induction of cell proliferation in the immune system, has an immunomodulatory effect as well as an immunostimulant that enhances cellular and humoral immune response (Maggini et al., 2007; Underwood and Suttle, 2001). In addition to being effective in the production of cytokines such as IL-1, IL-6 and TNF-α, zinc can also increase the immune response to phytohemagglutinin (PHA) and lipopolisaccharide (LPS) injections by showing comitogenic behavior (Wellinghausen et al., 1997). It has been also reported that the Zn level added to the diet in poultry affects the size of the lymphoid organs, and low Zn level causes a decrease in the weight of the lymphoid organs related to T-cell function (Kidd et al., 1996).

In addition to many studies on inorganic and organic Zn, there has been an increase in nutritional studies investigating the effects of Zn nanoparticles on the immune system and parameters in recent years. In a preliminary study conducted by Mahmoud et al. (2021), the addition of 10, 20 and 40 mg/kg ZnO nanoparticles to the broiler diet did not make a

significant difference in serum IgG, IgM and interferon (IFN)-γ concentrations between the groups. In an experiment performed by Eskandani et al. (2021) in broilers, control group was supplemented 70 mg/kg diet Zn-sulphate and other five experimental groups were supplemented 70 mg/kg Zn-aminoacid complex and 30, 50, 70 and 90 mg/kg Zn-nanoparticles, respectively, and no significant difference was detected in total immunoglubulin, IgG and IgM titers in terms of antibody titers against sheep red blood cell (SRBC) injection on day 35. In terms of antibody titers on day 42, no significant difference was found in total Ig and IgG titers, except for IgM titers. On the 42nd day of the study, the IgM titers were significantly higher in the group given 70 mg/kg Zn-amino acid complex and the groups given 30 and 90 mg/kg Zn-nanoparticle compared to the other groups. Also, there was no significant difference between the groups in terms of the relative weight of the lymphoid organs (b. Fabricius, spleen and thymus).

Antibody titers against New Castle Disease (NDV) and Avian Influenza Virus (AIV) were measured on the 35th day in broilers obtained from groups in which 50, 75 and 100 ppm nano ZnO injections were made in ovo on the first day of incubation in fertile broiler eggs, and there was no significant difference between the groups in terms of AIV antibody titers, monocyte, heterophile and lymphocyte counts, however, the total white blood cell count was significantly higher in broilers obtained from in ovo nano-zinc applied groups compared to the control group, and it was also determined that the antibody titers against NDV decreased depending on the nano-zinc level applied and were significantly lower than the control group (Biria et al., 2020). In an in ovo trial performed by injecting 20µL of saline solution containing 0.5 and 0.6 mg ZnO nanoparticles per 50 mL into fertile broiler eggs, Palouj et al. (2021) reported that there was no significant difference between the administrations in terms of antibody titers 7 days after vaccination against AIV and NDV vaccine made on the 28th day of hatching, but the antibody titers after 14 days showed a significant increase and in ovo nano-ZnO administration increased immunity in the blood. In addition to vaccination 28 days after hatching, no significant difference was found in the results of the inter-digital skin test performed to evaluate cutaneous basophil hypersensitivity (CBH) for cell-mediated immunity. In the same trial, the researchers also evaluated the relative gene expression levels of IL-2 and IL-12 and reported that injection of 20µL of saline solution containing 0.5 mg nano-ZnO in 50 mL led to the highest gene expression level and therefore this was the most appropriate nano-ZnO concentration. In another study performed in ovo, Jose et al. (2018) determined that in ovo administration of different forms of zinc (0.5 mg Zn sulfate and Zn methionine per egg in the two experimental

groups, 0.04 and 0.08 mg nano-Zn per egg in the other two experimental groups, respectively) did not cause a positive trigger on posthatch immune status in broilers and there was no significant difference between the applications in terms of cell-mediated immune response and humoral immune response. In an experiment on turkeys by Jankowski et al. (2019) to investigate the effects of the addition of conventional ZnO and ZnO nanoparticles to the per kg of diet separately as 100 (high), 50 (moderate) and 10 mg (low), the highest plasma IgY level was determined in 50 mg nano-ZnO group. While the level of IgM increased in response to the highest nano-ZnO dose, it decreased in response to the moderate nano ZnO dose, and no significant effect was observed on IgM level in response to the lowest nano-ZnO. Plasma IgA level was determined only in the highest nano ZnO group, while plasma IL-6 levels increased and decreased in the moderate and lowest nano ZnO group, respectively. Abedini et al. (2018a) reported that the use of each ZnO, nano-ZnO and Zn-methionine as Zn sources at a rate of 80 mg/kg diet in layer hens did not make a significant difference between Zn sources in terms of immune response to SRBC and PHA skin test, and all three Zn sources produced higher antibody titers in terms of relevant parameters compared to the control group. They also reported that the antibody titer against NDV in the nano-ZnO group was significantly higher than the control and normal ZnO group, and there was no significant difference between the groups in terms of the percentages of monocytes, lymphocytes, heterophiles/lymphocyte. In a previous trial in laying hens conducted by Tsai et al. (2016), the addition of 60 mg/kg nano-ZnO to the diet did not make a significant difference in terms of PHA skin challenge test, GRBC (goat red blood cell) antibody titer, IgG level and γ-globulin, did not produce an effective result in terms of immune response compared to the control group given normal inorganic ZnO at a rate of 40 mg/kg diet. In contrary, El-Katcha et al. (2017) determined that the addition of 60, 45, 30 and 15 ppm ZnO nanoparticles in broiler diets increased the percentage of lymphocytes, decreased the heterophile/lymphocyte ratio, improved phagocytic activity and antibody production against NDV compared to the group given inorganic ZnO, and they also reported that the use of ZnO nanoparticles in the diet at a rate of 45 mg/kg significantly increased the phagocytic index compared to its inorganic form. In a similar experiment conducted by the same researchers in 2018, similar results were obtained and tey reported that the addition of 60 and 30 ppm ZnO nanoparticles to the diet did not make a significant difference in the antibody titer against AIV in laying hens compared to the control group given 60 ppm inorganic ZnO, however, there was a significant improvement in phagocytic index, phagocytic activity and NDV antibody titer (El-Katcha et al., 2018a).

Effects of zinc nanoparticles on antioxidant activity

During the physiological respiration and metabolism processes in all living things consuming oxygen, a significant part of the oxygen is transformed into superoxide anion radicals and hydrogen peroxide, leading to the formation of endogenous free radicals. Antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), minerals such as Zn and Se, and some vitamins play a role in the elimination of these radicals (Altıner and Bilal, 2022). Reactive oxygen species (ROS), including peroxide, superoxide, hyroxy radical and singlet oxygen, are by-products of normal metabolism of oxygen and are present at low levels in normal cells (Hayyan et al., 2016; Herb et al., 2021). In addition, enzymes such as cytocrome p450, monooxygenase, nitric oxide synthase (NOS), xanthine oxidase, cyclooxygenase (COX) and lipoxygenase (LOX) cause ROS formation as a result of their own enzymatic reactions, and excessive formation of ROS leads to lipid peroxidation and damaging effects on proteins and nucleic acids (Jarosz et al., 2017). Zinc plays a role as a cofactor of SOD and CAT enzymes, especially for cytosolic and extracellular SOD enzymes, which provide the main antioxidant defense against ROS (Mariani et al., 2008).

The antioxidant mechanism of zinc is not fully understood. According to the examined chronic and acute effects, it was reported that long-term exposure of the organism to zinc caused the induction of metallothoneins with various antioxidant activities, chronic zinc deficiency increased susceptibility to oxidative stress, the increase in the level of zinc in a short time created sulfhydryl stabilization by protecting the sulfhydryls of certain enzymes from oxidation, and it reduced OH released from peroxide through antagonism of redox-active transition metals such as iron and copper (Powell, 2000). In addition to lipid peroxides (LOOH), reduced glutathione (GSH), oxidized glutathione (GSSG), SOD, GPx, CAT activities evaluated in the investigation of antioxidant status in studies, malondialdehyde (MDA) level is also another parameter evaluated. MDA, which is accepted as a marker of oxidative stress, is a product released as a result of peroxidation of lipids and its level in tissues is frequently measured to estimate the degree of lipid peroxidation (Davey et al., 2005). It was reported that the addition of Zn to the diet stimulated and increased the activity of Cu-Zn-superoxide dismutase, suppressed the formation of ROS, and therefore reduced the level of MDA (Nielsen et al., 1997).

In addition to peroxides, which are the first products of oxidation, hydrocarbons, aldehydes, ketones, alcohols and organic acids are also formed and these products negatively affect the nutritional value, sensory properties and shelf life of animal products (El-Massry et al., 2002). Consumer concerns about synthetic antioxidants such as butyl hydroxy

toluene and butyl hydroxy anisole, which have been used for a long time to control lipid oxidation in meat and meat products, have increased the number of studies on the search for alternative antioxidant sources (Botsoglou et al., 2002). In addition to numerous studies on the antioxidant effects of conventional and organic Zn mineral, the effects of Zn nanoparticles in this context have also been examined in recent years. The addition of 20 mg/kg ZnO nanoparticles to the diet in broilers increased serum SOD and CAT activity and total antioxidant capacity (Zhao et al., 2014). In a broiler study in which different zinc sources were compared, the addition of Zn-sulphate, Zn-methionine, Zn-nano sulfate, Zn-nano methionine and Zn-nano max (synthesized by nano chelating technology) to the diet at a concentration of 80 mg/kg showed a positive effect on lipid oxidation formed in tigh meat by significantly reducing the MDA concentration at 0 and 50 min. after lipid oxidation. Zn-nano methionine significantly reduced the MDA concentration compared to Zn-sulfate and Zn-methionine at the 100th minute, and compared to Zn-sulphate and Zn-nano sulfate at the 150th minute of oxidation (Mohammadi et al., 2015). In broilers, Eskandani et al. (2021) determined no significant difference between the control group, in which supplemented 70 mg Zn-sulphate per kg diet, and the experimental groups, in which supplemented 30, 50, 70 and 90 mg Zn nanoparticles, in terms of MDA concentration in leg meat at 48th hour and 60th day, however, they determined the lowest breast meat MDA concentration in the group supplemented 30 mg nano-Zn. In a study conducted by Lee et al. (2022) in broilers, it was reported that the addition of 80 mg/kg nano-sized hot-melt extruded Zn-sulphate to the diet significantly increased serum and liver SOD activity compared to the group given the same level of inorganic Zn-sulphate, and it was also reported that MDA levels decreased significantly only in groups supplemented with Zn without being affected by the Zn source compared to the control group.

It has been reported that coccidial infections can lead to an excessive increase in free radicals due to the increase in immune activity and an increase in serum nitric oxide and MDA concentrations due to increase in ROS production (El-Katcha et al., 2018b). In a study conducted in broilers infected with Eimeria spp. while MDA levels increased and antioxidant activity decreased significantly in the unsupplemeted group 4 weeks after infection compared to the uninfected group, the addition of 20 ppm ZnO nanoparticles to the diet caused a numerical decrease in MDA level and there was a statistically significant increase in antioxidant activity (El-Maddawy et al., 2022).

There are also reports that Zn nanoparticles produced by green synthesis have positive results in terms of antioxidant status. In a broiler experiment comparing three different Zn sources as inorganic ZnO,

green nano-ZnO and market nano-ZnO, the addition of 80 ppm market nano-ZnO and green nano-ZnO (synthesized from Catharanthus roseus) to the diet created significantly higher serum SOD, GPx and CAT level than the other groups, also, green nano-ZnO showed a significantly higher antioxidant effect following nano ZnO and lipid peroxidation was significantly lower in these groups (Dukare et al., 2021). Recently, in a trial carried out by Hidayat et al. (2021) in broilers to investigate the effects of nano-Zn (synthesized from Psidium guajava leaves by the green synthesis process and containing phytogenic compounds), SOD level in tigh meat was significantly higher as an indicator of high antioxidant activity, and MDA level was significantly lower as an indicator of low lipid peroxidation in groups supplemented with nano-Zn.

Conclusion

Nanotechnology, which is seen as a new approach that can be applied in all other technology and science branches, is also seen as a promising scope in the field of poultry nutrition and production. The production of zinc, which is an indispensable trace element among the minerals that have a special importance in poultry nutrition and plays an important role in the regulation of metabolic and cellular functions by participating in the structure of many biochemical enzymes, in nanoparticle sizes in accordance with nanotechnological developments has also made it necessary to conduct scientific research in this regard. In parallel with the fact that nanotechnological developments are still in their infancy and the research on this subject is limited, it is quite possible to see differences in the results obtained from the researches on the application of zinc nanoparticles in poultry nutrition. There are also study results where Zn nanoparticles have positive effects on performance parameters such as live weight gain, feed efficiency and feed consumption, especially in poultry, compared to other zinc sources naturally found in inorganic form in nature, as well as similar effects. However, its small size compared to their natural inorganic forms, larger surface area compared to their volume, higher bioavailability on target organs and tissues by direct absorption and less excretion from the digestive tract even at lower concentrations has been seen as remarkable advantages. In addition, the positive results on immunity, antioxidant status and meat and egg quality, as well as being cheaper than organic forms, increase the potential of Zn nanoparticles to take place in poultry nutrition. Therefore, in addition to the limited number of studies, more comprehensive and detailed new studies providing results are needed.

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Chapter 25

OF GALEN AND ANATOMY

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"physicians need anatomy to the highest degree"

Galen was the unquestionable authority in medical sciences from the 2nd to the 16th century A.D., and his writings and manifestations, just like Aristotle, had acquired the gravity of doctrines that could not be judged. He was the most fruitful writer of ancient Rome. A fire annihilated the most part of the Galen's writings and only 118 of 500 treatises could be saved. His works were translated in 22-volumes of books by C. G. Kuhn in the 19th century.

Ancient Greece and Predecessors of Galen

Ancient Greece was the centre for the art depicting and sculpturing human bodies in a superior esthetic fashion. Science of human anatomy was of remarkable attention. Hippocrates of Cos (460-375 B.C.), "the father of medicine" provided accurate descriptions of many parts of the body. "Hippocratic Corpus", a collection of his medical writings, included several references to anatomy. The Heart, pericardium, valves and great vessels are accurately and scientifically described for the first time by him.

Aristotle (384-322 B.C.), though not a pysician, was one of the most prominent predecessors of Galen, like him who to dissect animals. The anatomical studies of Aristotle based on a physiological background, due to his understanding that parts of human body had specific functions. His pupil Diocles performed dissections on human bodies, but he never did, still he examined human fetuses and, furthermore, made systematic analysis of animal bodies. He established the academic discipline of anatomy on scientific ground. He also contributed to the anatomical terminology. The terms he used and suggested are still in use in the today's medical and anatomical nomenclature. From Aristotle, Galen derived a keen sense of critical empiricism.

In the city of Alexandria, Egypt, founded by Alexander the Great and established by the mighty Ptolemaio Pharoahs, the physicians Herophilus of Chalcedon (Kadıköy today) and Erasistratus performed dissections on human bodies. Dissection of the human body was permitted for around 40 years during the days of Herophilus, thence abandoned again for the next 1800 years. Although he was blamed by Celsus (25 B.C. – 50 A.D.) to perform vivisection on humans, no certain evidence is found.

Herophilus and Erasistratus clearly identified the nervous system and circulatory system. It is believed that separate neural pathways had already been appreciated by them during their time. He claimed that damage to motor nerves induced paralysis. Herophilus named the meninges and

ventricles of the brain, diversed cerebellum and cerebrum and recognized that the brain was the “seat of intellect” and not a “cooling chamber” as stated by Aristotle. He for the first time described CNII, CNIII, motor portion of the CNV, CNVII, CNVIII and CNXII.

Herophilus was a pupil of Praxagoras of Cos (Bodrum Today), who had made significant contributions to Aristotelian anatomy by distinguishing arteries from veins. He thence progressed his mentor’s studies and following his recognized the importance of taking a patient’s pulse.

Rufus of Ephesus (80-150 A.D.) has a distinguished place in the history of anatomical sciences. He composed a lexicon of anatomy which was the first attempt.

Galen minds the classics of Hippocrates,and Aristotle. He uses the terms “the Ancients” and “the Greeks” as almost synonymous with those who are brilliant and excellent. He addresses his studies to timeless classics, so that he overreaches his particular “social and historical context.”. The contents of his treatises are more universal and pioneer raising fundamental questions. He denies referring himself as a member of any of the medical sects. Nevertheless, Hippocrates was a master for him, referring him as divine and he saw himself as completing and extending the master’s legacy.

Biography and Personality

Galen of Pergamum a.k.a Claudius Galenus was born in Pergamon (now Bergama, Turkey), in 126 AD, as the only child of Aelius Nicon, a wealthy and intellectual.architect. Pergamon was a very lively intellectual center comprising Smyrna, Ephesus, Rhodes, Cos and Miletus, which represented the highest in art and education in the world. The city had one of the best libraries among its contemporaries which had been greatly expanded by King Eumenes II.

Unlike his mother, Nicon was a concerned father providing him with a sound education. He tells:

“ I compared the excellence of my father’s disposition with the disgraceful passions of my mother, I resolved to embrace and love the former qualities, and to avoid and hate the latter.”

At the age of 14, he was referred by his father to a school of philosophy in Pergamon where he studied Platonic, Aristotelian, Stoic, and Epicurean systems for 3 years. In 145 AD, Asclepius, the god of healing, told Nicon in his dream, to allow his son to study medicine. Galen studied nedicine with the distinguished physicians at the Pergamum temple of Asclepius

for 4 years. In 148, when Galen was 19, his father passed away. This made young Galen an independent rich man. Galen's wealthy background forced him to travel in search of the best education. He spent 9 years travelling, learning, spending time at the Great Library of Alexandria which was the most famous and largest library and also a part of a research institute known as the "Museum" in Alexandria, Egypt. He continued his medical studies during these years first in Smyrna (now Izmir, Turkey), thence Corinth, Crete, Cilicia, Cyprus, and Alexandria. For Galen, the spur to anatomical knowledge originated in Alexandria. In 157, when he was 28, he came back to his native town where he was appointed surgeon for gladiators and the medical director at the temple medical school located outside the town near a mineral spring, from autumn 157 to autumn 161.

This four years experience achieved him invaluable practical medical knowledge of anatomy, trauma, and sports medicine. He had the opportunity to observe the effects of acute injuries to head and spine. From then on, Galen may have realized that Rome could offer the opportunities his talents and ambition demanded. In 162 AD, he moved to Rome. Galen rapidly had a reputation through his public lectures, anatomical demonstrations and proficiency as a physician in Rome where charlatans and inadequate surgeons were governing the medical community. This reputation brought him to the attention of the Emperor Marcus Aurelius. The emperor asked him to join them at their headquarters in Northern Italia, where they were engaged in military operations against Germans. Despite of his service as a surgeon he also discussed philosophy with Marcus Aurelius during the long campaign. The emperor appreciated Galen, and Galen profited from his fellowship. Emperor Marcus Aurelius called him the "best of physicians and the first of philosophers."

The Emperor and his family and rich families engaged to the emperor were not the only patients of Galen but he also treated ordinary people, slaves and peasants who applied to his clinic free of charge. He indeed was a popular public figure. He stayed as personal physician to Emperor Aurelius, his family and the empire's environment until his death.

Galen is thought to be medium-height person with a hawk nose and prominent chin. He often used strong rhethorics rooted from his brilliant education and wide knowledge to get people to agree with him. He was a top intellectual, nevertheless, an honest and a humble person. He criticizes his contemporaries for their ignorance, greed, and inadequate medical knowledge.

According to Galen, Empiricists and Methodists could not cover the sound value of anatomical investigation in everyday practice. Anatomical dissection and experimentation were not epistemologically validated

by the empiricists and the Methodists also did not find any need for the researches of anatomical sciences.

CLAUDE GALIEN

Anatomical Studies

Herophilus and Erasistratus claimed the importance of the Ventricles of the brain for the first time, however Galen described the anatomy of them in a detailed fashion. Galen describes lateral ventricles (the first and second, at right and left) of the brain in each hemisphere. He defines the communication between them and the third ventricle across the midline via an interventricular foramen. The third ventricle is stated by

him to communicate with the fourth ventricle via the cerebral aqueduct which was previously regarded as a ventricle by some authors. And the fourth ventricle communicates with the central canal of the spinal cord, consequently, he accurately defines the anatomy of the ventricules. Galen's ventricular system is a series of chambers, connected by canals, and communicating with the brain substance, the cranial nerves and the spinal cord. A fine network of veins and arteries are found in the lateral ventricules connecting to the optic nerves via pores. Galen addresses this as a discovery of the ventricular (lateral) origin of the optic nerves. He noted from a vivisection of a pig that while exposing the brain the pig continiued to blink its eyes even when dissecting a deep structure, the fourth ventricle. With this experiment he tried to prove that no physiologic relationship is found between the fourth ventricule and the eye. He attributes his skills in anatomical dissection. He cites the accurate writings of Herophilus and Erasistratus on the subject. He claims other anatomists not to able to understand this marvellous work of nature, however, he believed ventricles elaborated, stored and distributed psychic pneuma.

Galen discovered bilateral nerves on both sides of the neck coursing inferiorly toward the heart and then ascending back to the larynx. He stated :

"In the passage of the nerves across the thorax, a branch reascends on each side by the same pathway which it took before in descending; thus it accomplishes a double course.…It reascends from there to the larynx where the nerves insert themselves into the muscles in question. I call these two nerves the recurrent nerves (or reversivi) and those that come upward and backward on account of a special characteristic of theirs which is not shared by any of the other nerves that descend from the brain."

These findings with dissections made him very proud. He expressed his mighty feelings as:

"All these wonderful things, which have now become common property, I was the first of all to discover, no anatomist before me ever saw one of these nerves, and so all of them before me missed the mark in their anatomical description of the larynx."

"Now let me once and for all make this general statement to apply to my whole treatise so as not to be forced to say the same thing repeatedly: I am now explaining the structures actually to be seen in dissection, and no one before me has done this with any accuracy. Hence, if anyone wishes to observe the works of Nature, he should put his trust not in books on anatomy but in his own eyes and either come to me, or consult one of my associates, or alone by himself industriously practice exercises in dissection; but so long as he only reads, he will be more likely to believe

all the earlier anatomists because there are many of them."1 And he added: "I have continued my practice on until old age, and never as yet have I gone far astray whether in treatment or in prognosis, as have so many other doctors of great reputation."

Galen believed that the recurrent laryngeal nerves gained strength to close the vocal cords due to the exclusive design of a pulley system.

He discovered the decussating pattern of the fibers of the external and internal intercostals and thee clinical implications. During the vivisection, he cut the phrenic nerve and observed the function of these fibers. He additionally observed diaphragm's function after he cut the intercostal nerves at their emergence from the spinal cord.

Galen classified most of the cerebral structures. He recognized seven of the 12 cranial nerves, the cervical, brachial and lumbosacral plexuses and sympathetic ganglia. Knowing the functions of the muscles, he distinguished the origins of the muscles from the insertions. One of the legendary experiments of him was the demonstration of the function of the laryngeal nerves.

For Galen, anatomical knowledge provides information in examining psychic or physical activities and is a precision tool for the surgeon.

Galen summarized Marinus' twenty-volume text on anatomy and formed a four-volume book.

Table 1. Galenic Eponyms

Eponym	**The term**		
Veins of Galen	Internal cerebral veins deep cerebral veins	The two internal cerebral veins merge to form the great cerebral vein	These paired midline vessels arise at the interventricular foramina. They drain subcortical and periventricular structures as well as the choroid plexus.
The great vein of Galen	Great cerebral vein Vein of Galen	Deep cerebral vein formed by the basal veins of Rosenthal, the internal cerebral and some superior cerebellar veins. It is situated in the quadrigeminal cistern, posterior to the brainstem and third ventricle.	It runs downward to the splenium of the corpus callosum to drain into the straight sinüs.
Ampulla of Galen	Galen's ampulla	distal part of the great cerebral vein	Aneurysms of them are true aneurysms, although very rare

Galen's nerve	Galen's anastomosis	the direct connection between the dorsal branches of the internal laryngeal nerve and the recurrent laryngeal nerve	Thought to have an essential role in the innervation of the larynx, even though its function was not well manifested

Galen believed some of the blood from the heart went to the brain, where it was mixed with the third kind of pneuma which gives the brain its particular functions as well as flowing out through the nerves, enabling the functions of the muscles and to experience the external habitat by means of the sensory mechanism.

In the field of orthopaedics, writings of Galen base mostly on Hippocratic theories expressed in his treatises. In his "On Fractures and On Joints" this is obvious. Nevertheless, the Alexandrian traces in Galen's neuroanatomy of the spine could be easily figured out.

According to him, if the spine would be of much more smaller units, it could be more flexible however this flexibility would lead it to result in a more vulnerable form. The number of the vertebrae is ideal in order to prevent the spinal cord from damages. The anatomical details of the spine are magnificiently presented in his books. He accurately describes the vertebral and intervertebral foramina. He defines the posterior vertebral joints and emphasizes the role of them in preventing the vertebral column from hyperextension that may result in vertebral fractures and lacerations of the neighbouring arteries and veins.

The foramen ovale and the ductus arteriosus were recognized and defined by Galen. He figured out that these structures were peculiar to the fetal heart and that they undergo closure after birth.

Galen stated that the total transection of the medulla spinalis results in bilateral loss of sensation and mobility under the lesion level, whereas in a semi-transection of the medulla spinalis the related disorders are limited to the lateral half of the body.

He claimed that damage of the spinal marrow at first two cervical vertebrae or the following two, respiration terminates although the cardiac functions are not affected if transection is at the first cervical vertebral level. If the fifthe cervical vertebra is damaged the upper extremities will loose sensibility and motion. Galen also expressed that below the level of sixth cervical vertebra, paralysis of the muscles of intercostal spaces and abdomen, denervation of the intestines, vesica urinaria, and genitals and also paraplegia occur.

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Chapter 26

INFLAMMATION AND CARDIOVASCULAR DISEASE

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Inflammation and Cardiovascular Disease

The mechanisms involving immune system and inflammation in the pathophysiology of many disease states has been realized more in recent years. Immune system and inflammatory response is not limited to the defense mechanism of the body and therefore the scope is much broader than the infectious and rheumatologic diseases. The diseases in the subheadings of autoimmunity, hypersensitivity, immunosuppression are actually composing a very limited part of the health problems related with immune system. İnflammation is a reaction of the immune system to different reasons besides infections and includes complex interactions between immune cells. It has been better understood recently that chronic low-grade inflammation has a substantial role in the development a variety of chronic diseases including cardiovascular, neurologic, renal, hepatic diseases as well as diabetes, and cancer. The patients with chronic inflammatory diseases like rheumatoid arthritis, systemic lupus erythematosus have increased risk of cardiovascular morbidity and mortality. Moreover, previous reports about this subject have presented that inflammatory conditions such as periodontitis, some viral or bacterial infections, hypersensitivity reactions have also increased risk.[1]

Atherosclerosis

Atherosclerosis is one of the main mechanisms producing cardiovascular disease and related morbidity and mortality. Some recently defined triggers of atherosclerosis-such as disturbed sleep, physical inactivity, the gut microbiota, air pollution and stress have become more popular research subjects. Inflammatory pathways combine traditional and these novel risk factors.[2] The earliest lesions on vessel walls are called as fatty streaks, can be encountered in early childhood, are actually inflammatory lesions containing mainly macrophages and T lypmhocytes. Endothelial denudation and dysfunction following a process involving endothelial injury or repeated injuries provokes chronic inflammatory response which in turn, result in atherosclerotic lesions. Increased low-density lipoprotein levels as well as free radical producing insults such as cigarette smoking, diabetes, obesity, hypertension, genetic alterations, increased homocysteine levels, viruses or bacteria like Chlamydia pneumonia are some possible causes provoking chronic inflammation related endothelial dysfunction and following artheriosclerosis.[3] Injury results in an increase in the adhesiveness and permeability of the endothelium, which brings the inflammatory cells and platelets to the venue. Moreover, pro-coagulant properties of the endothelium start to dominate and production of vasoactive substances, inflammatory cytokines and other chemo-attractant molecules ascend. Continuation of exposure to the stressor or in other words if the fire cannot

be extinguished by the inflammatory response, which can be thought as firefighters, the fire will continue indefinitely. Mitogenic factors that are released from endothelium and smooth muscle myocytes as well as platelets and immune cells stimulate further activation of smooth muscle cells. Activated smooth muscle cell migrate and proliferate and together with the accompanying prothrombotic organization and increased collagen synthesis forms a more advanced lesion.[4] Collagen, elastin and proteoglycan synthesis increase in the extracellular matrix.. Macrophages and smooth muscle cells convert to foam cells and LDL-modification takes part in this step.[5] And if the event continues, more firefighters and probably army soldiers have to be involved in the fight which means macrophages and lypmhocytes will come to the area. These soldiers of the immune system use heavy weapons like hydrolytic enzymes, and also cytokines, chemokines, and growth factors that increase the local damage. This eventually results in necrosis and the formation of a fibrous cap over the advanced atherosclerotic lesion.[3]

A chronic, low-grade inflammatory process keeps attracting the cells of the immune system into this plaque. LDL with its core protein ApoB and Apo-B containing triglyceride-rich remnant particles serve as autoantigens within the plaque.[6] A positive correlation between the amount of antibodies against LDL oxidation epitopes and atherosclerotic plaque formation has been reported. Therefore, the mechanism behind Atherosclerosis includes chronic inflammation with a secondary autoimmune component.[7]

Oxidized-LDL is a marker of plaque inflammation. Reactive-oxygen species promotes LDL oxidation. Modifications of LDL provokes its ingestion by the macrophages at the injured vessel area. Moreover, oxidized phospholipids directly provoke inflammation at the arterial subendothelium by binding to Toll-like receptors (TLRs). TLRs are a form of pattern-recognition receptors that induce pro-inflammatory signaling. Cholesterol also causes formation of microcrystals in the endothelial cells which activate inflammasome. Inflammasome activation results in the activation of Interleukin-1β (IL-1β) which is a key cytokine that stimulates plenty of pro-inflammatory cytokines.[7]

CANTOS, a randomized, double-blind trial of canakinumab which is a therapeutic monoclonal antibody targeting interleukin-1β, showed that canakinumab significantly decreased the rate of recurrent cardiovascular events compared to placebo. This result was independent of any lipid-lowering effects. However, fatal infections were more frequent in the canakinumab arm owing to the depressive effects on immune system.[8]

Antigen presentation triggers the CD4+ T-helper cell activation which is one of the considerable steps in atherosclerosis. Many type of cells including macrophages, dendritic cells, and B cells function as antigen presenting cells (APCs) for both effector and memory-T-cell responses. Signals and cytokines from the APCs at the inflammation site determine the direction of the immune response. Moreover, B cells can secrete cytokines that can promote atherogenesis by producing GM-CSF to induce pro-atherogenic TH1 immunity. B-regulatory cells secrete IL-l0 and pose anti-inflammatory action.[7]

Stress and inflammation

Psychological stress is associated with increased inflammation and it is an independent coronary artery disease risk factor. Previous reports presented associations between major inveterate psychologic stress sources as work stress, social isolation, depression, dementia, and increased inflammation.[9]

Acute stress events were also shown to stimulate inflammatory pathways. The mechanism behind this interaction is low-grade systemic inflammation. Tumor necrosis factor (TNF)-α and interleukin (IL)-1β are two principal pro-inflammatory cytokines that take role in inflammatory process of atherosclerosis and they promote the secretion off secondary cytokines like IL-6. TNF-α and IL-6 are two pro-inflammatory cytokines that were proved to predict the risk of coronary artery disease.[10]

Cognitive perception of a life event assigns the stress response to the situation. The instance is perceived as stressful if it is interpreted as stringent or minatory by the individual and exceeds his or her perceived coping capacity. Therefore, perception of the stress is more important than the stress itself and varies among individuals. A chronic stress originating from job strain owing to insurmountable job expectations and perception of insufficient control on job issues and also working for long hours were shown to increase the risk of coronary artery disease significantly. Living childhood period in a low socioeconomic environment was associated with a increased mortality risk from coronary artery disease. Similar results were reported for social isolation, loneliness and vital exhaustion. Stress-related significant elevations in TNF-α, IL1β and IL-6 levels without a prominent increase in CRP were concluded in previous studies. Acute stress induces DNA binding of the transcription factor NF-κB (NF-κB-BA) that results in upregulation of mRNA levels of inflammatory cytokines as IL-1β and IL-6. Chronic stress results in decreased parasympathetic activity and inhibition of the cholinergic anti-inflammatory pathway besides causing a long-term increase in stress hormones including epinephrine, norepinephrine, dopamine, cortisol which eventually induce the formation

of pro-inflammatory cytokines and may have more prominent effects on TNF-α and CRP, as well as IL-6.[11]

Smoking and inflammation

Smoking is related with atherosclerosis and it is an independent predictor of new coronary artery lesion formation.[12] Both active and passive smoking results in vasomotor dysfunction. Decreased NO availability results in an impairment in endothelium-dependent vasodilation which is an early change in smokers' vessels. İncrease in VCAM-1, ICAM-1, E-selectin levels, expression of adhesion molecules as CD11b/CD18 on monocytes some other detected changes related to smoking. Moreover, higher serum cholesterol, low-density lipoprotein (LDL) and triglyceride levels and lower high-density lipoprotein levels are detected in lipid metabolism of smokers. Lipid peroxidation and autoantibody titers to oxidized LDL are also augmented. Platelet dysfunction and altered thrombotic balance in favor of pro-thrombotic state are added to these inflammatory changes. Smoking tar, macrophages and neutrophils, endogenous sources of reactive oxygen species like eNOS are sources of free radicals in smokers. Superoxide increase will result in a decrease in NO availability.[13]

Diet and inflammation

A healthy diet which contains more fibres, vegetables and fruits reduces cardiovascular disease risk. Previous studies focused on some components of healthy diet as omega-3 fatty acids. They may inhibit the synthesis of pro-inflammatory cytokines, such as TNF-alpha, IL-1, and IL-2 and decrease expression of adhesion molecules on the endothelium. Higher intake of omega-3 and omega-6 fatty acids can alleviate the inflammatory response, decrease CRP levels and coronary artery disease risk. Cholesterol rich diet increases CRP levels whereas a diet composed of less saturated fat and less cholesterol is associated with a reduction in CRP as well as less arterial stiffness. Similarly, a fiber-rich diet composed of more vegetables and fruits have prominent antioxidant and anti-inflammatory effects. Higher fiber content in the diet and arginine-rich foods like nuts are related with lower CRP levels. Refined and processed grains cause acute hyperglycemia which impairs endothelium-dependent vasodilation, similar to the early effects of smoking. Moreover, short-term acute hyperglycemia due to refined carbohydrate intake may increase circulating levels of free radicals and peroxynitrite augmentation triggered by increased superoxide levels, protein Kinase-C and NF-kB increases the secretion of pro-inflammatory cytokines, such as IL-6, IL-18, and TNF-alpha as well as increasing CRP levels. Also Mediterranean diet has the potential to decrease the levels of IL-6 and CRP approximately 20%. It is possible to decrease the risk of acute coronary syndrome more

than 50% by this diet. Arrangement of the life style with a healthy diet approach usually increases the plasma adiponectin levels which is an anti-inflammatory protein produced by adipose tissue. Higher adiponectin levels are associated with a lower risk of myocardial infarction.[14]

Fried meat consumption also provokes inflammation and increases the pro-inflammatory cytokine levels such as TNF-α, IL-10, and IL-1β levels besides negatively affecting microbial gut flora. This harmful effect of fied food on the gut microbiota results in intestinal endotoxin production and provokes systemic inflammation.[15]

Gut microbiota which consists of more than 100 trillion micro-organisms is affected from the diet content and can be associated with systemic low-grade inflammation. Lower microbial diversity in gut microbiata composition is observed in obesity, diabetes, dyslipidemia and high systemic blood pressure. Predominance of gram-negative bacteria in gut flora results in production of endotoxins including lipopolysaccharides with pro-inflammatory actions. LPS can interact with Toll-like receptor 4 (TLR4) and by passing from the gut into the circulation, it can activate mononuclear cells causing production of IL-1, TNF-α, and IL-6. Another important product of gut microbiata is Trimethylamine-N-oxide (TMAO) which is produced mainly by *Clostridia* and *Enterobacteriaceae* from the degradation of carnitine, lecithin and choline.[16] TMAO has pro-inflammatory roles as increasing TNF-alpha and IL-1B, and inhibiting the production of anti-inflammatory cytokines such as IL-10. It has been shown that elevated TMAO levels are associated with increased risk of cardiovascular events.[17]

Sleep and Inflammation

Studies of self-reported sleep duration show that both shorter and longer sleep duration are associated with increased cardiovascular mortality. The MORGEN study concluded that insufficient sleep duration and poor sleep quality increase the total cardiovascular disease and coronary heart disease incidence.[18] Many studies reported increased levels of TNF-alpha, IL-6, or CRP in sleep deprivation. Sleep deprivation may result in imbalance in sympathetic and parasympathetic systems as vagal withdrawal which triggers inflammatory response. Moreover, less than normal sleep may result in metabolic disturbance including insulin resistance which also has pro-inflammatory consequences.[19]

Air pollution and inflammation

Short- and long-term exposure to air pollution increases the risk of cardiovascular morbidty and mortality. The particulate matter (PM) of the pollutant is a combination of solid and liquid particles. Their size

has different effects on human health. PM_{10} with a diameter <10 μm can reach the lungs and $PM_{2.5}$ with a diameter <2.5 μm can penetrate deeper. Exposure to PM_{10} and $PM_{2.5}$ induces sustained oxidative stress and inflammation resulting in adverse cardiovascular outcomes. The direct effects of air pollution may provoke acute cardiovascular responses such as acute coronary syndrome and arrhythmias. The indirect effects can promote systemic inflammatory response causing vascular dysfunction and atherosclerosis. Oxidative stress originating from air pollutants activates transcription factors like NF-kappaB and AP-1 which upregulate the expression of pro-inflammatory mediators as cytokines. Moreover, PM2.5 exposures may result in increased blood pressure through NAD(P)H oxidase- and eNOS dependent ROS generation which activates the Rho/ROCK signaling pathway. Activation of TLR4 and NADPH oxidase in monocyte/macrophages by oxidized phospholipids by the effects of these pollutants induces systemic inflammation.[20]

Not only the outdoor pollution but also the household air pollution affects the cardiovascular health negatively. Stoves may form a source of indoor pollution which results in higher concentrations of $PM_{2.5}$ which is associated with increased CRP levels. 25% increase in personal $PM_{2.5}$ was associated with a 10.5% increase in CRP.[21]

CD32 expression with dust fungi, monocyte expression of CD11 with PM2.5 in the bedroom, and house dust bacteria, CD31 expression and microvascular dysfunction due to dust were also reported.[22]

Exercise and Inflammation

Exercise has a complex set of effects of inflammatory pathways. Response to acute and regular exercise and the intensity od the exercise evokes pro and anti-inflammatory response. Moreover, inflammatory reaction differs according to the tissue type. High intensity endurance exercise causes increased levels of plasma IL-6 which was released to the circulation, starting with the neutrophilic and macrophage infiltration of the muscle. However, this does not cause a generalized immune response but rather turns into anti-inflammatory effect. One of the main long term effects of regular exercise is seen in adipose tissue by production of adipokines.[23]

	Muscle tissue	Adipose tissue	Vessels
IL-6	↑	↓	↓
TNFalpha	↓	↓	↓
M1 macrophage	↓	↓	
M2 macrophage	↓	↑	
IL-1	↓	↓	

Nitric oxide	-		↑
Metalloproteinase			↓
Fibrosis	↓	↓	↓
CRP		↓	

Figure 1: Main inflammatory changes during exercise[23]

Exercise causes IL-6 secretion by muscle tissue via a TNF-independent pathway. IL-6 stimulates the other anti-inflammatory cytokines such as IL-1ra and IL-10. IL-1ra inhibits IL-1β signal transduction and IL-10 inhibits the synthesis of pro-inflammatory cytokines such as TNF-α.[24] IL-6 starts to increase soon after the onset of exercise. However, unlike the endotoxemic inflammatory response, TNF-α and IL-1β production don't accompany the exercise induced IL-6 increase. Therefore, although exercise seems to share some pro-inflammatory pathways, net effect is anti-inflammatory. Epinephrine increases highly during exercise and it antagonizes TNF- α. Moreover, IL-6 increase as a response to exercise induces an increase in cortisol which is a well-known anti-inflammatory agent. Regular exercise results in blunting in IL-6 production both at basal levels and when stimulated by exercise. Moreover, both sedentary life style and associated abdominal adiposity provokes systemic low-grade inflammation which in turn increases cardiovascular risk. Furthermore, this inavtivity causes re-distribution of fat tissue to become more prominent intraabdominally. Interleukin-15 upregulation is prominent in the muscles of trained people. IL-15 has anabolic eefcts as stimulating myogenic differentiation and also it helps decreasing the amount of white adipose tissue. This lipolytic effect of IL-15 is also potentiated by IL-6. Knowing the atherogenic properties of inflammatory cytokines such as TNF- α and IL-1β which are decreased with regular exercise, the importance of exercise on chronic low grade inflammation to prevent cardiovascular diseases can be better interpreted.[25]

Certain anti-inflammatory peptides, metabolites, and RNA species are termed as exerkines which are released in response to exercise. Irısin, adiponectin, IL-6, IL-1ra are some exerkines that pose potential therapeutic targets for atherosclerosis and cardiovascular diseases.26

Summary

Chronic low-grade inflammation seems to be the main mechanism in the pathogenesis of severe diseases that are the most common reasons of morbidty and morality. Cardiovascular diseases, cancer, neurologic disorders, and many other common and severe diseases need a broader research related to the underlying inflammatory mechanisms. Better understanding of these mechanisms may form potential therapeutic targets

and moreover, open many paths for the primary and secondary prevention an even eradication of these diseases.

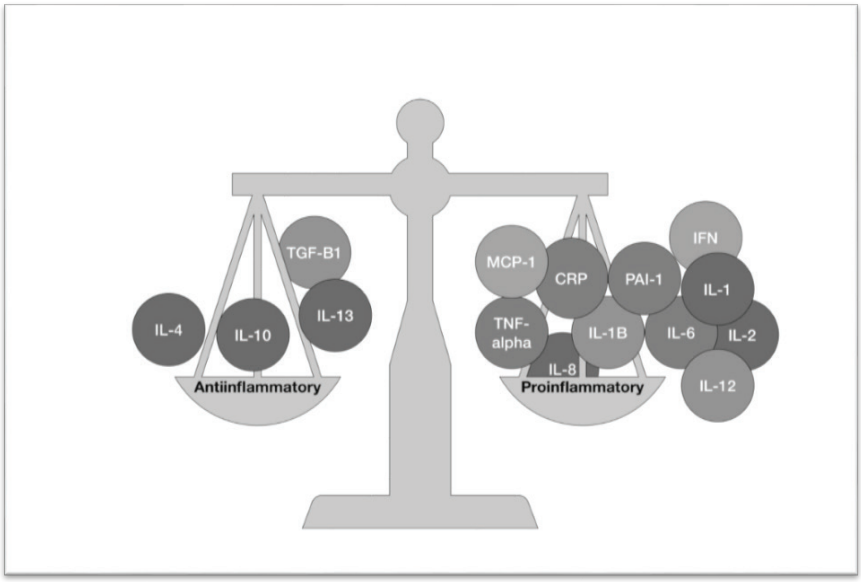

Figure 2. Summary of anti-inflammatory and pro-inflammatory cytokines involved in chronic low-grade systemic inflammation involved in the pathogenesis of cardiovascular diseases

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