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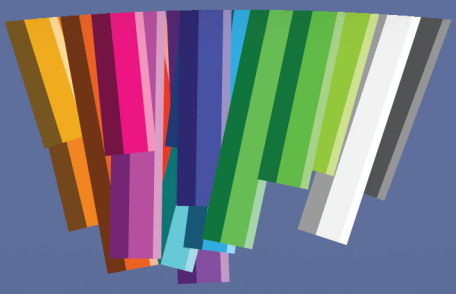

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Research & Reviews in Health Sciences

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CONTENTS

Chapter 1

CARBON DIOXIDE ABSORBENTS

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INTRODUCTION

The circle system and carbon dioxide (CO_2) absorbents have been clinically used in modern anesthesia in order to minimize the wastes generated during the use of volatile anesthetics. The CO_2 absorbent, which is contained in the rebreathing anesthetic systems, provides the elimination of CO_2 by chemical means. In respect of the use of volatile anesthetics, which have negative effects on global warming and cause environmental pollution, it is very important to manage the fresh gas flow (FGF) rate (1, 2).

During the use of the circle system, the anesthesiologist can control the percentage of the rebreathed gas by adjusting the fresh gas flow (FGF) rate.

1. HISTORY OF THE CARBON DIOXIDE ABSORBENTS

In the history of anesthesia, the studies on CO_2 absorption date back to the beginning of the 1900s. The "to and fro" system, which involves carbon dioxide absorption, was first used in the clinical practice by Alfred Coleman (1822-1902) (3). In 1903, Dragerwerk developed a device in order to absorb carbon dioxide from rebreathing gases using soda lime (mostly calcium hydroxide with some sodium and potassium hydroxide added). This device was used underground by the miners, who used oxygen cylinders. During these times, limewater and soda were investigated in terms of their CO_2 properties (4). In 1906, Franz Kuhn defined the two-compartment respiratory system involving potassium hydroxide (KOH) with the aim of eliminating $CO_2$from the expired gasses. Nonetheless, this system was not used in clinical practice due to reaction of KOH with chloroform, resulting in the formation of phosgene. In 1915, Wilson obtained soda lime, a CO_2 absorbent with high efficiency that is still used today (5). In 1923, Ralph Waters introduced a respirator involving an absorbent between the reservoir bag and the fresh gas inlet (6). In 1930, Brain Sward defined the circuit with CO_2 absorption (7).

Anesthesia circuits can be classified as follows:

Open system; open mask, insufflation (e.g. ether anesthesia

Semi-open systems; Mapleson's A, B, C, D, E, F systems

Semi-closed systems; Circle system

Closed systems .

Circle system

It is called the circle system due to the circular arrangement of its parts. It is a closed system, and is considered as a semi-closed system when operated with high gas flow. The use of the circle system involves the administration of gas and anesthetics to the patient under low resistance during inspiration and expiration, low back breathing, retention and removal of carbon dioxide.

Parts of the Circle System

The parts that make up the circle system, which is the most common respiratory system, are as follows:

- Fresh gas entry
- Unidirectional valves
- **Carbon dioxide absorbent**
- Respiration tubes
- Reservoir bag
- Y-piece
- Mask

The device that is made of single or double glass or metal and used in semi-closed and closed systems is called an absorber. Absorbent is a granular formation that absorbs and removes the carbon dioxide contained in the absorber.

Absorbent granules are placed in one or two canisters that fit tightly between metal parts consisting of the top and bottom parts. The use of double canisters enables good CO_2 absorption, replacement of the absorbent at less frequent intervals, and less gas resistance. Canisters vary in volume between 250-1000 mL. Canisters provide protection of absorbents. They have filter systems that prevent the inhalation of absorbent powders by the patient. They are produced in a transparent form in order to allow the external detection of the change of color in the absorbent (8).

2. CARBON DIOXIDE ABSORBENTS

Disadvantages of open and semi-open systems include causing intense air pollution, increased gas consumption despite the low amount of use, thereby increasing the cost, and the inability to heat and moisten the anesthetic gases (9,10,11). These negative aspects of open and semi-open systems necessitated the development of semi-closed and closed systems. In closed and semi-closed systems, the carbon dioxide in exhaled gases is absorbed and reused.

2.1. CO_2 absorption capacities of the absorbents

This can be described in two ways: According to the first one, CO_2 absorption capacity of the absorbent refers to the time required for the *(Inspired Minimum Carbon dioxide)* $IMCO_2$ pressure to reach the level of 2 mmHg (12,13). The second one involves the amount of CO_2 in liters, which can be absorbed by 100 g of absorbent (9).

Strong bases such as sodium hydroxide (NaOH), potassium hydroxide (KOH) have been added to the absorbent as a catalyst to increase the reaction speed and CO_2 absorption capacity. The CO_2 absorption capacity of the absorbent is directly proportional to the strong base concentration. A good absorbent is expected to have high absorption capacity with low toxic reaction rate with the volatile anesthetics, and to have low resistance to the air flow.

The amount of CO_2 in the air inhaled by the patient is monitored regularly by capnography.

In low-flow anesthesia, the increase in the rate of rebreathing would increase the use of absorbent, increasing the absorbent costs. As the flow decreases, less gas would be discharged out of the system; therefore, there is less elimination of exhaled gases. When the absorbent is exhausted in the system, the CO_2 concentration significantly increases due to the greater volume of rebreathing, and causes the risk of hypercarbia. Monitoring the amount of CO_2 inspired and expired is important in terms of patient safety (14).

Exhaustion of the absorbent refers to the loss of CO_2 absorption capacity in the absorbent dur to the transformation of the alkali hydroxides into carbonates (15). The durations of use are affected by the amount and content of the absorbent used, the size and moisture of the surface area, the amount of CO_2 exhaled by the patient, and the fresh gas flow (FGF) rate (16). The absorbent should be replaced when there is a change of color by 50-70%.

Reduction of the fresh gas flow rate (FGF) during general anesthesia lowers the costs by reducing the consumption of volatile anesthetics and the absorbent, and decreasing the contribution of the environment to greenhouse gas pollution.

Hendrickx et al. obtained novel data enlightening the challenges experienced in measuring the differences in performances of various products as well as the absorption capacities of the CO_2 absorbents. Hendrickx et al. analyzed the package life of the CO_2 absorbents, which had been prepackaged, firstly on Aisys (GE Medical) and recently on Zeus (Draeger Medical) application systems. They reported that it was

necessary to prefer absorbents with relatively lower costs and the longest duration of efficiency, which are able to reach a FiCo2 of 0.5% at the longest period (17,18).

In modern practice, where capnography is accepted as a standard monitoring system, longer duration of use would be obtained by using CO_2 as a guideline to replace the absorbent instead of any change in the color of the indicator. Absorbents, which minimize or eliminate strong bases, do not interfere with volatile anesthetics, and are clearly desirable for patient safety. Water is found as a thin film on the granular surface of the CO_2 absorbent. Absorbents with lower water content are exhausted quickly while there is an increased absorption rate and decreased adhesion and resistance in absorbents with higher water content (16).

Indicators that change color with acids or alkali are added to the absorbents. The most common indicator is the ethyl violet. Ethyl violet (CAS-2390-59-2), which is added into soda lime and baralyme, is a triarylmethane dye with a critical pH of 10.3. Other indicators used include phenolphthalein, Clayton yellow, ethyl orange and mimosa (12,15,19). These indicators change their colors as hydroxides are neutralized and transformed into carbonates. When the absorbent is fresh (pH: 12), its pH is above the critical level (pH: 10.3). As a result of the reaction of the white absorbent with CO_2, the pH level of the absorbent decreases ($pH<10.3$), and it turns into purple. When the absorbent turns completely purple, it is an indicator that it has lost its efficiency. On the other hand, the ethyl violet does not always indicate the functional state of the absorbent. This color change is usually reversible, as alkali hydroxides regenerate when no absorbent is used. The granules, which have lost their efficiency, are may turn back to their original colors when they are maintained unused; however, there is no significant improvement in their absorption capacities (15). Drying, which refers to the loss of water, and exhaustion, which indicates CO_2 absorption, should be distinguished from each other. Both drying and exhaustion may cause the change of color in the absorbent (15).

The change of color in amsorb plus, which does not contain strong base, is irreversible. On the other hand, the purple color of Drägersorb Free turns into dur to the regeneration of NaOH when no absorbent is used (12).

Carbon dioxide absorbents can be in the form of granules or half lentils. As the size of the granule gets smaller, the absorption surface increases, and more CO_2 can be absorbed, whereas the reistence to the gas passing through the canister increases. Certain dimensions have been

determined maintaining the optimum granule size. For instance, the most frequently used size is 4-8 mesh (0.32 - 0.64 cm in diameter).

In clinical practices, the absorbents are replaced based on the change in the color. It has been reported that this method is subjective, the change in the color of the absorbent should not be depended on alone in the clinical practice, and that the patients should also be monitored in terms of their findings related to CO_2 accumulation (increase in blood pressure followed by a decrease, acceleration of the heart rate, deep breathing and increased $PaCO_2$, sweating) (20).

3. CO_2 ABSORBENTS THAT ARE CURRENTLY USED

Absorbents with different names have been obtained by changing the contents and proportions of the CO_2 absorbents that are currently used.

Traditional CO_2 absorbents and premium CO_2 absorbents

$CaCO_3$, a catalyst that reacts with carbon dioxide for generating water and temperature, $Ca(OH)_2$ and water are used in all carbon dioxide absorbents. Within this cyclic process, $Ca(OH)_2$ is constantly re-moistened until it is completely exhausted or turned into $CaCO_3$ during the exothermic reaction of CO_2 emissions (21).

3.1. Traditional absorbents:

3.1.1. Soda lime

Carbon dioxide absorption is an exothermic chemical reaction. It refers to the neutralization of an acid by a base. Soda lime *contains* 80% of calcium hydroxide [$Ca(OH)_2$], 15% of water (H_2O), 4% of NaOH, 1% of KOH, and 0.2% of silica. Silica increases the hardness of the granules, and decreases the inhalation of the NaOh powders (9,11,19). Soda lime is produced in a granular form, which are also porous. Therefore, a wide absorption surface has been created where the gasses can pass through. Granules, which are able to pass through a sieve of 4-8 *mesh*, are used in the clinical practice of anesthesiology. The absorption surface gets wider as the granule diameter of the absorbent gets smaller. However, this is inconvenient as it would increase the resistance to air flow (19).

One hundred grams of soda lime absorbs 23 L (max 26 L) of carbon dioxide. An average absorbent absorbs 10-15 L of carbon dioxide per 100 g in a single canister system and 18-20 L in a double canister system. Since a normal adult emits 12-18 L of carbon dioxide per hour, 1 kg of soda lime can be effective for about 8 hours.

CO_2 absorption by soda lime is a chemical process, rather than a physical process. This is the neutralization of an acid by a base. Carbonates and water are released as a result of this neutralization.

$CO_2 + H_2O \rightarrow H_2CO_3$

H_2CO_3 + 2NaOH (KOH) → Na_2CO_3 (K_2CO_3) + **2 H_2O** + **ısı** (rapid reaction)

H_2CO_3+$Ca(OH)_2$ → $CaCO_3$+ **2 H_2O** (slow reaction)

Na_2CO_3 (K_2CO_3) + $Ca(OH)_2$ → $CaCO_3$+ 2 NaOH (KOH)

During this exothermic reaction, 1 mol of water is consumed; and 2 mols of water and 13.7 kilocalories of (kcal) thermal energy is generated as a result of 1 mol of CO_2 absorption. As a result, the CO_2 that has been included in the patient circuit is absorbed by the absorbent, while the system is moistened and heated (19,22).

Table 1. The absorbents that are currently used, and their contents (23):

Manufacturer	Name of the product	H_2O%	NaOH%	KOH%	$Ca(OH)_2$%	Other determinant	Usage in the USA
Allied Haeltcar Chemetron	e Baralyme	11.0 - 16.0	0.0	<5	73	$Ba(OH)_2$	None
Allied Haeltcare	Carbolime	12.0 - 19.0	3	0.0	>75	-	Yes
W.R Grace and Company	Sodasorb	15.0 - 17.0	3.7	-	50 - 100	-	Yes
Intersurgical Ltd.	Intersorb Plus	13.5 - 17.5	2.6	0.0	81	-	Yes
Intersurgical Ltd.	Spherasorb	13.5 - 17.5	1.3	0.0	78	4% Zeolite	Yes
Intersurgical Ltd.	LoFloSorb	13.5 - 17.5	0.0	0.0	78	6.5% Silica	Yes
Armstrong Medical Ltd.	Amsorb	13.5 - 16.5	0.0	0.0	79 - 82	$CaCl_2$	None
Armstrong Medical Ltd.	Amsorb Plus	13.0 - 18.0	0.0	0.0	>80	$CaCl_2$	Yes

Dräger Medical, Inc.	Drägersorb 800	16	2	3	75 - 83	$CaCl_2$	None
Dräger Medical, Inc.	Drägersorb 800 Plus	16	1 - 3	0.003	75 - 83	-	Yes
Dräger Medical, Inc.	Drägersorb Free	14 - 18	0.5 - 2	0.003	74 - 82	$CaCl_2$	Yes
Airgas/Molecular Products	Soda lime	15	<3.5	2.6	>80	-	Yes
Molecular Products	Sofnolime	12 - 19	<3.5	0.0	75 - 80	-	None
GE Medical / Molecular Products	Medisorb	12 - 19	<3.5	0.0	75 - 80	-	Yes

3.1.2. Baralyme

Baralyme contains 73% $Ca(OH)_2$, 20% $Ba(OH)_2$, <5% KOH, 11 - 16% water. Unlike soda lime, water binds to the molecule chemically. Thıs, it does not lose its moisture easily in a dry atmosphere. Baralyme has a pink color, and it turns purple by reacting with CO_2 (22).

Chemical reactions formed with baralyme :

$Ba(OH)_2 + 8\ H_2O + CO2 \rightarrow BaCO_3 + 9\ H_2O$ + temperature

$9\ H_2O + 9\ CO_2 \rightarrow 9\ H_2CO_3$

$9\ H_2CO_3 + 9\ Ca(OH)_2 \rightarrow CaCO3 + 18\ H_2O$ + temperature

KOH and NaOH have been blamed the most for the development of undesirable effects and reactions. For this purpose, it was assumed that undesirable by-products can be reduced by reducing the amount of both bases (5,24-27). Drägersorb 800 plus, intersorb, medisorb, spherasorb and sofnolime have been used among the absorbents produced for this purpose.

3.1.3. Drägersorb 800 plus

Drägersorb 800 plus *(Dräger, Luebeck, Germany)* was launched to the marked in 2000. It is hemispheric, white and odorless. It does not damage the ozone layer. It contains 75-83% $Ca(OH)_2$, 1-3% NaOH, 14-

18% H_2O, and 2.9% KOH. The $Ca(OH)_2$ powders it contains may cause irritation in the respiratory tract (7).

In an in vitro study, which analyzed the reactions of three different absorbents (amsorb, sodalime, drägersorb 800 plus) with sevoflurane at 45 °C in low-flow anesthesia, it was demonstrated that the compound-A concentrations, which were formed with soda lime and drägersorb 800 plus were ten times more compared to *amsorb* (28).

3.1.4. Amsorb

As a result of the continued search for a safe and economic CO_2 absorbent with a long life cycle, amsorb, loflosorb, superia , which contained no NaOH and KOH, was developed (5).

In 1999, Amsorb *(Armstrong Medical Ltd.*, Coleraine, North Ireland) was launched as a novel absorbent, which did not contain KOH and NaOH, and which did not react with sevoflurane and desflurane . It contains calcium hydroxide, calcium chloride, calcium sulphate and water. The absorption capacity of Amsorb is lower compared to soda lime (25), and has an efficiency equivalent to 40-90% of the efficiency of soda lime (12). Its most important advantage is that it does not contain hydroxides such as NaOH and KOH. The absence of these strong bases prevents the formation of toxic products such as CO and compound-A by reducing the volatile anesthetics (27).

3.2. Premium Absorbents

3.2.1. LiOH

It has been reported to be inert in reducing inhalation agents under humid or dry conditions. On the other hand, it is not recommended for use in clinical conditions due to its corrosive and caustic effects (5,24).

$LiOH + H_2O \rightarrow LiOH{\bullet}H_2O$

$2LiOH{\bullet}H_2O + CO_2 \rightarrow Li_2CO_3 + 3H_2O$

$2LiOH + CO_2 \rightarrow Li_2CO_3 + H2O$ (11)

LiOH has been reported as the absorbent with the poorest anesthetic agent reduction (24).

3.2.2. Memsorb

Memsorb (*Memsorb, DMF Medical, Halifax,* Nova Scotia, Kanada) is a recently produced absorbent, it does not react chemically with CO_2. It does not have any reactions with volatile anesthetics (29).

4. UNDESIRED EFFECTS OF CARBON DIOXIDE ABSORBENTS

Absorbents react with volatile anesthetics, and cause the formation of undesired effects and products (5,25). These are:

1- Retention and reduction of inhalation anesthetics

2- Formation of methanol, formaldehyde and formic acid

3- Increased temperature and fire

4- Formation of compound A-E

5- Formation of CO

Volatile anesthetics react with all CO_2 absorbents. Absorbents in order of reaction with volatile anesthetics are as follows: baralyme>soda lime>KOh-free soda lime> CaOH lime . The reaction is affected by the fresh gas flow rate, shape of the anesthesia circuit, content of the absorbent, inhalation anesthetics and its concentration, the temperature and moisture content of the absorbent (5).

4.1. Retention and Reduction of Volatile Anesthetics

It is important that carbon dioxide absorbents are not toxic, and that toxic products are not released when exposed to anesthetics. Strong bases such as sodium hydroxide (NaOH) and especially potassium hydroxide (KOH) are blamed for the breakdown of volatile anesthetics. Absorbents are absorbed by volatile anesthetics, leading to cost increases and delays in induction with volatile anesthetics. Anesthetic agents that contain difluoromethoxy, such as isoflurane (-CHF_2) cause the loss of anesthetics as a result of reduction by the dry CO_2 (24). Sevoflurane forms reduction products by reacting with CO_2 absorbents (30). Volatile anesthetics in order of reduction by the absorbents are as follows: sevoflurane> isoflurane>desflurane (31). Baralyme reduces sevoflurane four times more compared to soda lime (25). The reduction reactions between the volatile anesthetics and absorbents are exothermic, and the reactions with sevoflurane are particularly energic (32).

4.2. Formation of methanol, formaldehyde and formic acid

C*annizzaro* reaction resulting from the reaction of sevoflurane and CO_2 absorbent.

(**Figure** 1) Methanol causes the formation of formaldehyde and formic acid. The temperature that is formed due to this reaction further increases the reaction rate (25). Methanol and formaldehyde are flammable gaseous compounds (5).

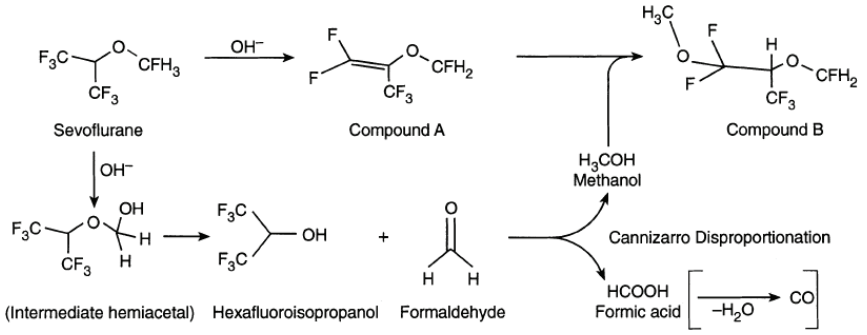

Figure 1. *Cannizzaro reaction*

4.3. Temperature increase

It has been reported that increase temperature and fire most frequently occurs due to the use of sevoflurane with baralyme or soda lime (32). Baralyme used to be quite popular. It has been used safely for long years; however, excessive temperature (400 °C) may occur due to the reaction of dry baralyme that has lost its moisture and sevoflurane, and it may cause ignition and fire (31). Baralyme was withdrawn from the market by the manufacturer. The reaction of dry absorbents with sevoflurane may cause excessively high temperature. The byproducts such as formaldehyde, methanol and formic acid may lead to fire in the surrounding anesthesia circuit rich in oxygen/nitrogen (25,28).

The temperature of the absorbent is affected by the metabolic condition of the patient, CO_2 elimination, fresh gas flow rate and ventilation (33). The increase in temperature resulting from the reduction of sevolflurane with soda lime has been found to be three times greater compared to the reactions of desflurane and isoflurane (34).

4.4. Formation of compound A

Compound A (fluoromethyl-2-2 difluoro-1-(trifluoromethyl) vinyl ether) is a product of destruction resulting from the reaction with CO_2 absorbents during sevoflurane anesthesia.

Factors causing increase in the concentration of compound A are as follows:

- Low fresh gas flow rate or use of closed circuit
- Long term administration of anesthesia
- High concentration of sevoflurane
- Use of baralyme (more than soda lime)

- High temperature of the absorbent
- Type of the absorbent (content of NaOH and KOH)
- The water content of the absorbent (the drier the soda lime, the more volatile anesthetics it will absorb)

The temperature of the absorbent and the reduced fresh gas flow increases the amount of compund-A (25). Accordingly, Food and Drug Administration (= FDA) reported that it would be safe to use 2 MAC/hour of sevoflurane in a minimum of 1 L/min fresh gas flow rate. Nonetheless, there is no limitation of fresh gas flow in many countries (12,27).

The metabolites of Compound A are nephrotoxic, rather than itself (5). Until now, Compound A has been demonstrated to be nephrotoxic in rats (28). On the other hand, the nephrotoxicity of Compound A has not been demonstrated in human (5,23,35).

4.5. Formation of carbon monoxide (CO)

The drying of the absorbents increase the formation of CO. Gases, which are left free in the anesthesia device between cases and outside of work, may pass through the breathing circuit and dry the absorbent. The level of carboxyhemoglobin may increase up to 30% or higher concentration levels. Production of CO in the anesthesia circuit is based on the inhalation agent, type of the absorbent, temperature, moisture and fresh gas flow rate (5,24,25 30,32,36).

The production of CO based on the reaction of the volatile anesthetics with the absorbents is as follows: desflurane>enflurane>isoflurane>halothane=sevoflurane (25,30). The reaction of sevoflurane with dry absorbent is exothermic. The destruction accelerates with the increase of temperature, and the production of CO increases (30). Significant CO production with the reduction of sevoflurane can be observed when the ambient temperature exceeds 80 ^{o}C (32).

Since it was understood that carbon monoxide production depends on the presence of alkali hydroxides in the absorbents (36,37), the new absorbents have been developed containing NaOH without KOH, or containing only $CaOH_2$ without strong alkali hydroxide (24).

In low flow anesthesia, the moisture of the absorbent is preserved, and the formation of CO is prevented (38). High fresh gas flow rate reduces the amount of water generated by CO_2 absorption, and dries the absorbent (19,32,33,39). High temperature increases the production of CO.

The cases, in which carbon monoxide poisoning cases are reported, are mostly the cases where anesthesia is applied on Monday. Especially

if the gas flow continues, the dry gas outlet passing through the absorbent dries the absorbent over the weekend, and this causes this clinical outcome.

In a 24 year-old female patient, SpO_2 decreased to 93%, HbO_2 decreased to 63%, and COHb decreased to 36% during the 5% desflurane anesthesia in the presence of baralyme. The patient completely recovered after the replacement of baralyme immediately (25).

5. STRATEGIES FOR REDUCING THE UNDESIRED EFFECTS OF ABSORBENTS

The CO_2 absorbents are indispensable substances in the clinical practice of anesthesia. Nonetheless, drying and heating due to uncontrolled use may cause severe morbidity and mortality (25).

In clinical practice, it is not known when the CO_2 absorbents would dry due to exposure to fresh gas flow (40). It was argued that soda lime dried critically after 42 hours with a fresh gas flow of 7 L/min (41). It was reported that the change of color insoda lime was not apparent even after drying for 60 days, and that monitoring the change of color would be poor indicator

for drying (15).

Generally, the time of replacement for the absorbents is determined according to the change of color in the clinical practice. It has been reported that this method is subjective, and the patient should be monitored in terms of CO_2 accumulation in the clinic (25).

In routine clinical conditions, the amount of byproducts formed due to the interaction of the absorbent and volatile anesthetics, whether the absorbent has dried, and the temperature of the absorbent cannot be determined. Therefore, it is necessary to know the properties of the absorbents, and to organize the user manuals in terms of patient safety.

The properties expected from an ideal absorbent are as follows: It should not be toxic (harmful effect), it should not react with frequently used anesthetics, it should not have low resistance to air flow, it should be easy to use, and it should be cost effective (26).

In the report of the APSF conference held in 2005 on the safety of carbon dioxide absorbents and the review published in 2006, the following recommendations were made for the “prevention of the drying of the absorbents in clinical practice” (23,25):

1- After the administration of anesthesia, all gas flows should be turned off when the device is not used, and especially at the end of the day.

2- If the device has not been used for a while and you are not sure about the moisture of the absorbent, the absorbent should be replaced routinely regardless of the color indicator.

3- If the gases are left free at night or at the weekend, the absorbent in both chambers should be replaced.

4- The absorbent should be replaced regularly every Monday morning.

5- The absorbent should be replaced when there is a change of color due to exhaustion.

6- If a compact canister is used, it should be changed more frequently.

7- If there is overheating in the carbon dioxide absorbent chamber, the absorbent should be changed, and the patient should be monitored for CO poisoning.

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Chapter 2

RECOMMENDATIONS FOR THE PREVENTION AND CONTROL OF AEROSOL CONTAMINATION DURING DENTAL PROCEDURES IN THE NOVEL CORONAVIRUS PANDEMIC

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Since the outbreak of the novel coronavirus disease (COVID-19), due to the strong transmission capacity of the virus and the long incubation period and the characteristics of human-to-human transmission, there may be asymptomatic infections. In the case of poor protection, the nosocomial infection rate is as high as 41% (Wang et al., 2020). At present, the main transmission routes of virus that have been identified are respiratory droplet transmission and close contact transmission. In the case of exposure to high concentrations of aerosol in a relatively close distance, there is also the possibility of aerosol transmission, which is related to dental procedures and oral cavity (Ge, Yang, Xia, Fu, & Zhang, 2020). Droplets and aerosols produced during dental procedures as the source of infection for potentially spreading diseases have attracted the attention of researchers in the 1960s (Micik, Miller, Mazzarella, & Ryge, 1969). If dental staff perform dental procedures on patients with incubation period or asymptomatic COVID-19 infection without their knowledge, the use of high-speed rotary instruments or ultrasonic treatment equipment during procedure will generate a large number of droplets and aerosols containing pathogenic microorganisms, causing indoor air contamination (Harrel & Molinari, 2004; Szymanska, 2007). It poses a serious threat to the health of dental workers and patients. Therefore, in addition to strict prevention and control of infection, the routine patient care in dental settings should be done in a scientific and orderly manner. In this review, the control and prevention of aerosol formation and spread in dental clinics during the COVID-19 pandemic is discussed. Also, recommendations for psychological support for patients and dental stuff who expose to aerosol generating procedures were also included.

FORMATION AND SPREAD OF AEROSOLS DURING DENTAL PROCEDURES

The broad concept of aerosol refers to a colloidal dispersion system formed by solid or liquid particles suspended in a gas medium, with a particle diameter between 0.001 and 100 μm. It was first reported the existence of bacterial aerosol particles in dental clinics and the relationship between these aerosol particles and the health of dentists and patients in the 1960s (Micik et al., 1969). Researchers divide the air particles in the dental settings into aerosols and droplets. Among them, aerosol particles have a diameter of <50 μm, can be suspended in the air for a long time and have extremely strong penetrating power, and are considered to be the most likely to be inhaled into the lungs and spread diseases. Droplets mainly refer to particles with a particle size> 50 μm, which stay and suspend in the air for a relatively short time.

Due to the characteristics of dental procedures, high-speed rotating instruments and ultrasonic equipment are often used in the dental health care. As the high-speed rotary instruments rotates and the ultrasonic scaler vibrates and resonates, the compressed air and water are aerosolized during the cycle operation, which will produce a large amount of aerosols and droplets containing saliva, nasopharyngeal secretions, blood, and pathogenic microorganisms (Acharya, Priya, Purohit, & Bhat, 2010; Holbrook, Muir, Macphee, & Ross, 1978). At present, a large number of studies have confirmed that aerosols and droplets are generated during dental procedures, especially when high-speed rotary instruments and ultrasonic equipment are used to operate, the bacterial content in the clinic aerosol is significantly increased, which has become a source of infection for potentially transmissible diseases.(Bennett et al., 2000; Cristina et al., 2008; Harrel & Molinari, 2004)

THE ROLE OF AEROSOLS IN TRANSMISSION OF INFECTION

The distance traveled by aerosol particles has a negative correlation with the diameter of the particles. The larger the diameter of the aerosol particle, the faster the particle settling speed, and it can settle on the surface of the object in a shorter time. Conversely, the smaller the particle diameter, the slower its settling speed, which can be suspended in the air for several hours and evaporate to form a droplet nucleus with a smaller particle size, with microbial particles as the main component, and then diffuse with the movement of the airflow (Kohn et al., 2003). It was showed that the aerosol in the air in the dental clinic spread faster, in a closed environment, the contamination range of aerosol can spread almost to the entire clinic, including the non-operation area (Grenier, 1995). Another study found that the dentist's exposure to aerosol-contaminated air for 15 minutes resulted in inhalation of 0.014 μL of aerosol. In severe cases, they could inhale 0.12 μL aerosol within the same time (Bennett et al., 2000). It should be noted that when the patient's oral cavity contains normal microbial colonies, the aerosols and droplets produced during oral treatment are not enough to cause serious danger. However, when the patient's oral cavity or nasopharynx carries blood-borne virus, respiratory virus, the aerosol particles that contain a large number of microbial nuclei can cause serious risk to both doctors and patients (Bennett et al., 2000; Cristina et al., 2008). It is now clear that a variety of pathogenic microorganisms, including the SARS (SARS) coronavirus of the same group as the new coronavirus, can be transmitted through droplets and aerosols (Ge et al., 2020; Yu et al., 2004). In view of the large spread of the novel coronavirus, the long incubation period and atypical symptoms,

some asymptomatic infections may be the reasons why the infection spreads very quickly and easily. Therefore, any dental procedure that produces aerosols can lead to the spread of the pandemic, which brings great challenges for providing dental health care during the prevention and control period.

PREVENTION AND CONTROL STRATEGIES OF AEROSOL CONTAMINATION IN DENTAL CLINIC

Prevention of movements of patients with incubation period or asymptomatic infection in the clinic

Research on the spatial distribution of aerosols in the dental office shows that the concentration of aerosols and droplets is the highest within two feet (0.609 m) centered on the patient (Harrel & Molinari, 2004). If the patient is infected during the incubation period or asymptomatic, the movement or activity in different areas of the hospital during the treatment process is regarded as a mobile source of infection for the transmission of pathogenic microorganisms. Therefore, it is necessary to minimize the risk of transmission of the novel coronavirus in ambulatory dental clinics, and strict in-hospital and intra-department secondary pre-examination, triage, screening and registration should be implemented for patients and accompanying persons (Meng, Hua, & Bian, 2020). During the diagnosis and treatment process, except for removing the mask during treatment, the patient is required to wear a mask throughout the rest of the time. After diagnosis and treatment, necessary precautions should be taken to keep the distance and frequency of movement of the patient within the hospital as low as possible.

Personal Protection Precautions

It is recommended that dentists and dental assistant who perform aerosol generating procedures during the pandemic should adopt three-level protection and be able to correctly hold the order of wearing protective equipment (Ge et al., 2020): Hand wash, wear a disposable cap and a face mask (N95 or KN95) and use the positive pressure method to check the air-sealing of the mask (Ge et al., 2020), wear lining gloves, wear protective suit, wear protective goggles or face shield, hand disinfection, wear latex gloves or nitrile gloves (outer layer); Sequence of removing protective equipment: Hand disinfection with quick-drying disinfectant, change gloves, hand disinfection, take off protective goggles or face shield, take off the outer gloves, wrapped off protective clothing, hand disinfection, take off the inner layer of gloves, hand disinfection, take off your cap and mask, hand disinfection, take off work clothes,

wash your hands (running water). When leaving the operation room, you need to take off protective masks, glasses, protective clothing and other protective equipment. Note that when removing the mask, the ear straps or headband should be tightened to make it leave the face in a stable state, and avoid rough removal that causes the flipping of the mask to cause disease-causing particles to splash or float in the air. Carry out personal hygiene after get off work, and pay attention to the cleanliness of the nasal cavity and ears.

Prevention and control of aerosol contamination during dental procedures

The use of pre-procedural mouth rinses: Instruct patients to gargle with 1% povidone-iodine mouthrinse for 3 minutes to reduce the number of microorganisms in droplet aerosols generated by dental procedures, and instruct patients to seal the mouth with the mouth of a disposable water cup as much as possible, and spit the gargle.

Four-handed dentistry: The implementation of four-handed dentistry in dental treatment can improve the quality of medical care and work efficiency, minimize the exposure time, and reduce the incidence of hospital cross-infection (Villani, Aiuto, Paglia, & Re, 2020). Dental assistants need to be familiar with procedure routines, and have complete preparations before treatment to avoid getting in and out of the clinic during treatment, and reduce contamination and cross infection.

Applying rubber dam isolation: For the teeth that require spray treatment, rubber dams should be routinely isolated after local anesthesia, and 75% ethanol cotton balls should be used to disinfect the teeth in the operation area to reduce quantity of pathogenic microorganisms in the aerosol generated during the operation. It is recommended to use a 3D rubber dam without punching, which is more convenient and quicker to operate.

Use high volume evacuation equipment: Studies have shown that when using high-speed rotary instruments or ultrasonic equipment to treat teeth, working with a high-volume evacuation device throughout the procedure can help reduce the generation of droplets and aerosols, and reduce the number of bacteria contained in aerosols in the dental clinic (Day, Sandy, & Ireland, 2006; Sawhney et al., 2015). The straws connected to the high volume evacuation device have different models and specifications such as elbow straight head and flat mouth type. Among them, the elbow and straight mouth diameter is about 2.5~5.8mm, and the flat mouth type has a larger mouth diameter, about 8~15mm, so it is recommended to use a flat-mouth strong straw for operation as much as possible.

The prevention and control of the waiting area: Improve the rigid isolation between the waiting area of the department and the treatment area. The waiting area should have good ventilation conditions, adopt a continuous ultraviolet air circulation disinfection machine, and sterilize with ultraviolet radiation (1h, at least twice a day) in an unmanned state. It is recommended to make appointments at different times to reduce attendants and other measures to avoid gathering of people in the waiting area, and to shorten the waiting time of patients. All waiting patients and companions are required to wear effective masks correctly, and the waiting distance between patients is > 1m. Those who do not wear a mask or do not meet the requirements should provide a disposable medical surgical mask and guide them to wear it correctly. The triage nurse regularly inspects the waiting area, understands the patient's condition and the implementation of mask wearing, etc., and maintains a good waiting order. Every day at noon and after the afternoon shift, the waiting chair and the floor of the waiting area should be wiped and disinfected with a disinfectant with an effective chlorine content of 1000mg/L at least twice a day.

The prevention and control of the treatment area: It is recommended to choose an independent clinic with well-ventilated light and equipped with air disinfection equipment such as ultraviolet air circulation disinfection equipment as a special clinic for aerosol generating procedures. Thoroughly clean and tidy up the treatment room before each use. Cleaning includes thoroughly cleaning the ceiling, wall, desktop and floor of the consulting room. The medical equipment and equipment used are stored in a closed locker. For large equipment that is difficult to move, such as dental operating microscopes, it is recommended to wrap and protect it with a protective bag.

Before the clinic is used, it should be reviewed by a hospital-sensing expert on the spot, and the prevention and control measures against aerosol contamination should be strictly implemented during the use process, including: (1) Place a floor mat soaked with 2000mg/L chlorine disinfectant at the entrance of the aerosol generating procedure clinic, and update it every 4 hours. The floor mat should be kept moist. (2) Keep the windows open for ventilation and continue to use the ultraviolet air circulation sterilizer to sterilize the state (3) After the clinic is used, the windows should be closed and disinfected with ultraviolet air circulation disinfection machine and ultraviolet radiation for 1 hour, and then open the windows for 30 minutes to ventilate for use. (4) Use ultraviolet air circulation disinfection machine and ultraviolet radiation to strengthen disinfection after each shift for 1h.

PSYCHOLOGICAL INTERVENTION ON AEROSOL TRANSMISSION DURING THE PANDEMIC

Psychological intervention of the patient

Informing the patient correctly: As a public health emergency, COVID-19 has a serious impact on the public's psychological state. The administrative departments of the countries have widely promoted COVID-19 related knowledge and basic prevention and control measures through the media, newspapers and the Internet, and the public has a wide range of awareness. With the in-depth understanding of the diagnosis and treatment of COVID-19, the aerosol transmission route of the novel coronavirus has become a hot issue of public concern. Dental care providers can inform patients about what is aerosol and its knowledge of the characteristics and prevention in the spread of diseases. On the one hand, patients can correctly recognize the characteristics of dental procedures, and avoid too contempt or over-stressed psychology of dental care consultation and during the pandemic period; on the other hand, enable patients to correctly understand it also pays attention to the formation of aerosols in the dental setting and its potential health hazards during the pandemic of respiratory infectious diseases.

Psychological intervention for patients with dental emergencies: Priority should be given to online consultations about dental care services during the pandemic period. Dentists should attempt to reduce the number of emergency dental incidents in the institution through online consultation with their professional knowledge. However, the treatment of patients with dental emergencies may also be delayed due to operating conditions, or their over-sensitivity to aerosol contamination, and even show resistance to dental staff during treatment. Emotions have an adverse effect on disease control. Hippocrates once pointed out that doctors mainly use two methods to treat diseases, one is medicine, and the other is language. Especially in special times, medical staff should take on the role of psychological helpers, change treatment concepts and methods in time, and give personalized suggestions from the perspective of comforting patients' emotions, so as to reduce the impact of psychological factors on oral emergencies (Day et al., 2006). Inform patients of the risk of consultation during the pandemic period, and emphasize the effective protection measures taken by hospitals and departments to reduce the risk of infection during the patient's consultation, especially for aerosol transmission, so as to avoid the patient's resistance due to lack of cognition or excessive panic, and gain the understanding and cooperation of patients and their families.

Psychological intervention of dental staff

A large number of studies have confirmed that when high-speed rotating instruments and ultrasonic equipment are used for aerosol generating procedures, the microbial content in the aerosol in the clinic is higher than that of other groups that do not use rotating and ultrasonic equipment (Rautemaa, Nordberg, Wuolijoki-Saaristo, & Meurman, 2006). Therefore, under the influence of the COVID-19 pandemic, dental professionals should know that the high-speed rotating instruments have become a source of high-speed spread of disease by aerosols and droplets of pathogenic microorganisms. If patients with latent or asymptomatic infections are treated without knowing it, aerosol particles containing pathogenic microbial nuclei will cause indoor air contamination, posing a hazard to the health of dental workers and patients.

The new coronavirus is extremely contagious, and there is currently no effective treatment for COVID-19. Although dental care workers are not front-line personnel in the fight against the pandemic, when faced with patients who are troubled by acute toothache and need to open pulp drainage and other aerosol generating procedures, dentists still face difficulties such as high risk of infection and high psychological pressure (Harrel & Molinari, 2004). Therefore, dental hospitals and departments should earnestly care about the physical and mental health of dental staff, carry out mental health assessments of dental staff, strengthen psychological assistance measures, and carry out targeted psychological adjustments and psychological interventions to reduce the psychological burden of dental staff.

Only correct understanding can help to eliminate inner fear and anxiety and get better at work. As medical staff, dentists and dental assistants have the support of professional medical knowledge. At the same time, hospitals and departments also need to continuously organize learning and update related prevention and control knowledge methods and skills about COVID-19 and aerosols in accordance with the documents issued by the reliable national or international health authority organizations, such as CDC or WHO, so that dental staff can rationally and scientifically understand the aerosols role in the spread of COVID-19, and be proficient in protection knowledge and proper use of protective equipment. At the same time, colleagues are encouraged to support and care for each other, and relieve psychological pressure in a timely manner to ensure that medical staff have a good psychological and mental state, and orderly perform medical services during the pandemic.

CONCLUSIONS

To sum up, as long as we strictly follow the guidelines for infection control in the dental setting, the aerosols and droplets produced during the dental prosedures are not enough to cause serious danger, and there is no need to panic about it. However, during the current period of the Covid-19 pandemic, if the patient's mouth or nasopharynx carries the pathogenic COVID-19 virus, the aerosol particles that contain a large number of microbial nuclei can cause serious risk to both dental stuff and patients. Therefore, for patients who really need to perform aerosol generating procedures during the COVID-19 pandemic, firstly, pre-inspection, triage and screening should be strictly performed; secondly, through the rigid isolation of the waiting area and the treatment area, to ensure that patients are diagnosed and treated first. Strengthen environmental disinfection; finally, apply the rubber dam four-handed operation to forcefully attract and standardize the diagnosis and treatment process, and at the same time do personal protection and psychological counseling. Thereby reducing the risk of cross-infection, strictly controlling the spread of the pandemic, and ensuring the safety and health of dental stuff and patients to the greatest extent.

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Chapter 3

EXERCISE HORMONE IRISIN: THE RELATIONSHIP OF COVID-19 AND INFLAMMATION

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Introduction

Coronavirus 2 (SARS-CoV-2) which causes severe and rapid respiratory disease symptoms outbreak started in December 2019. By this time, it has caused 2.95 million deaths in the world. Covid-19 cases increasingly continue. Covid-19 is reported as an international emergency and public health problem by the World Health Organization (WHO). The virüs effects people in various ways. Covid-19 complaints vary from person to person. Many people get through this disease mildly symptoms such as sore throat , headache, loss of taste and smell..There is no need for hospitalization Some patients applied to the hospital with complaints of cough, fever and shortness of breath. Some of these may be hospitalized. People infected with the virus begin to show symptoms within an average of 5-6 days. However, this period can take up to 14 days. Coagulation may be outlined in some Covid-19 patients. Researches show that hypertension, cardiovascular, type 2 diabetes, chronic obstructive pulmonary and obesity patients possibility to become Covid-19 high compare to healthy individuals [1]. Angiotensin converting enzyme 2(ACE2) is a important protein for the development of Covid-19 [2]. ACE2 is expressed in various organs such as kidneys, central nervous system, cardiovascular system, gut, adipose tissue and especially lungs [3]. SARS-CoV-2 that causes Covid-19 is using ACE as a receptor to enterance the lung [4].Obese and diabetic individuals may become targets for Covid-19. The reason is that the increased adipose tissue and ACE2 level in these people turn this tissue into a viral reservoir [5]. Practicing physical activity is crucial to prevent Covid-19. Performing physical activity, regularly in our houses reduces the risk of capturing coronavirus [6,7].It has been also demonstrated to reduce depression prevalence and to enhance mental health [8]. Obesity is very important health problem in the world and makes people more sensitive to severe infection by SARS-Cov-2. This virus expecially targets the adipocytes and lung cells[9]. Covid-19 mortality and morbidity is higher in many metabolic and chronic individuals such as obesity, T2DM, hypertension, cardiovascular diseases, respiratory tract and cancer [10]. Various studies have showed that obese people have increased inflammation level and BMI, which is closely related to Covid-19 [11,12]. Research on America people most of the obese people is related to severe COVID19 [13,14]. In a New York City research , 3,615 people were diagnosed Covid-19 positive , 21% of these people have a BMI between 30-34 kg / m2 and BMI of 595 patients is over 35 kg / m2[15]. In a study conducted in Mexico and Italy, diabetes mellitus and obesity improved the risk of Covid-19 infection [16,17]. Severe inflammation related with rised risk of mortality in coronavirus patients. Covid-19 patients generally have proinflammatory cytokines

activation such as Interleukins, IFN, and C-reactive protein. Obesity is classic inflammatory disease. During obesity these inflammatory marker levels are increased in the adipose tissue, serum, liver, skeletal muscle and lung [18,19]. Coactivators and corepressors are important molecules for transcription factors (TF). TF should have coactivators and corepressors to realize their functions. Transcriptional coactivators bind to TF and support their activation but do not have specific DNA sequences on their own. The PGC-1 family, which is one of the best-known families of transcriptional coactivators, has three known members (PGC-1α, 1β and associated protein (PRC) [20]. Peroxisome proliferators activated receptor-γ coactivator-1α (PGC1-α) is stimulated by energy metabolism and produced in muscles by exercise. PGC1-α mediates various biological programs [21], such as biogenesis. Also, high levels of PGC 1-α prevent muscle damage. PGC1-α stimulates the expression of several muscle gene products. These are interleukin 15 (IL-15), leucine-rich glycoprotein 1 (LRG1), tissue inhibitor matrix metalloproteinase 4 (TIMP4), vascular endothelial growth factor (VEGF) and fibronectin type III domain containing protein 5 (FNDC5), precursor irisin [22]. FNDC5 was discovered by 2 different groups in 2002. Fibronectin type III domain containing protein 2 (FRCP2) and peroxisomal protein (PeP) are other names for FNDC5 [23]. The FNDC5 protein includes 209 amino acids (aa). FNDC5 protein contains 29 aa signal peptide, 94 aa fibronectin domain, an unknown part with 28 aa, after that 19 aa transmembrane and C-terminal 39 aa, respectively [24]. FNDC5 is a kind of type 1 membrane protein. This protein is proteolytically cleaved from the N-terminal domain by an unknown enzyme and 112 aa irisin occurs and is released into the bloodstream. FNDC5 is a precursor of irisin and is expressed from high to low in the following organs and tissues [25].

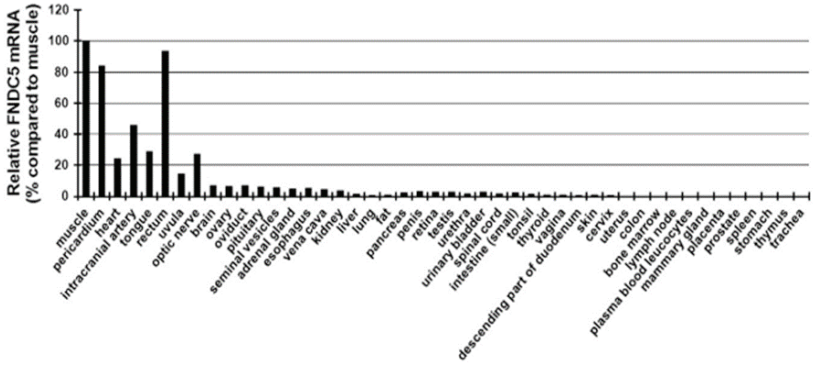

Figure 1. *Distrubution of FNDC5 in varies tissues and organs[25].*

Skeletal muscle is a crucial organ for humans and comprises myocyte cells [26]. Myokines are secreted from myocytes during or immediately after exercise from skeletal muscle. Myokines regulate metabolic situations in diverse tissues and organs; for example, in the liver, brain, muscle and fat tissue [27,28]. Bostrom et al. reported a novel myokine that is released by muscle cells called irisin with a weight of 112 aa of 12 kDa. Irisin stimulates changes in white and brown adipose tissue. Compared with humans and mice, irisin is 100%, insulin 85%, glucagon 90% and leptin 83% similar to each other [21]. Irisin is a exercise myokine that causes energy consumption by changing white to brown adipose tissue [26]. Secretion after cleavage of irisin is similar to growth factors that epidermal and transforming. FNDC5 increases the levels of brown fat tissue genes (Elov13, Cox7a and Otop1) and on the other hand decreases synthesis of the white adipose tissue product leptin. A small amount (about 20 nM) of FNDC5 increases the expression of UCP1 7-1500-fold and increased UCP1 expression reduces ATP synthesis and causes heat production. The mechanism of irisin is summarized in Figure 2.

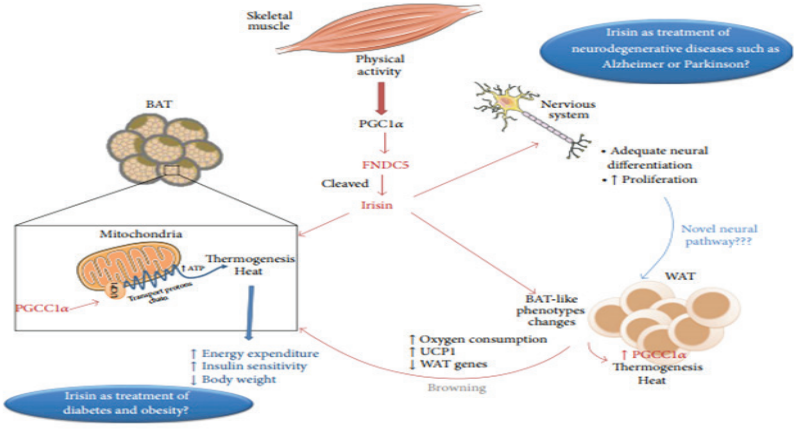

Figure 2. *The mechanism of irisin [29].*

In light of all this data, it can be said that FNDC5 regulates thermogenesis activation of brown adipose tissue [21]. Until now, no receptor was identified. Recent investigations indicated that irisin acts by binding to integrins which are members of the alpha v integrin family. Integrins are receptors that recognize soluble ligands and bind to extracellular matrix ligand [30]. These transmembrane receptors are responsible for cell adhesion, migration, and aggregation [31]. Irisin is produced in various tissues, such as adipose tissue, heart muscle, kidney, liver, myelin sheath, neural cells, optic nerve, over, Purkinje cells,

rectum, salivary glands, stomach, testis, tongue, and intracranial arterie [32]. The tissue with highest synthesis of irisin is skeletal muscle called myokine, but immunohistochemical studies have demonstrated that it is extensively synthesized in myocardium. Immunoreactivity of irisin was found in the parathyroid, submandibular, and sublingual glands. Irisin is also synthesized in fat tissue so it is stated to be an adipomyokine [33]. In this review, it is aimed to explain the relation of irisin, known as exercise hormone, with covid19 and inflammation.

Irisin and Inflammation Relation

Adipose tissue and muscle that were previously considered metabolically passive have been shown to play significant role in metabolic regulation through secretion of a number of hormones and hormone-like peptides, such as leptin, ghrelin, nesfatin, preptin, adropin and irisin. These peptides are described to communicate with cells in an autocrine (the cells that produce them)/paracrine (nearby cells), or in an endocrine manner (distant tissues). Myokines are mainly secreted in skeletal muscle and adipokines are in adipose tissue. The myokines described in the literature that are additionally known to be secreted by adipocytes are termed as adipo-myokines [34]. Three adipose tissues were defined as beige, brown and white in mammals. White adipose tissue stores energy as a triglycerides (TG) but brown and beige adipose tissues are privatized in energy expenditure and adaptive thermogenesis [35].White adipose tissue is the main source of adipokines with pro- and antiinflammatory properties, including leptin, adiponectin, interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) [36].Adipokines are secreted both from adipocytes and other cells of the adipose tissue, such as endothelial cells and macrophages [37]. Obesity, especially visceral adiposity, is characterized by a state of lowgrade systemic inflammation. Proinflammatory adipokines and molecules secreted from adipose tissue are implicated as the cause of increased cardiovascular disease risk, development of insulin resistance and so-called metabolic syndrome associated with obesity. Obesity is a chronic disease and closely associated with inflammation. It was identified to increase fat mass and cause growth of adipose tissue [38]. Since irisin was accepted as a thermogenic agent, it was considered to play a role in reducing fat mass in obese patients. In a study about irisin, the level of irisin was found to be higher in men and obese people [39]. FNDC5 mRNA in skeletal muscle and irisin levels in blood decreased in individuals who had undergone bariatric surgery six months after the operation. It was concluded that energy restriction and surgically induced weight loss decreased the level of irisin [25]. In other studies, the level of

irisin decreased in diabetes patients compared to controls [40]. An increase was found in the milk and blood of mothers with gestational diabetes [41].Sesti et al. examined circulating irisin and cardio-metabolic variables in their study. They determined insulin sensitivity and common carotid intima-media thickness (vascular atherosclerosis indicator) and showed that there was a negative relationship between insulin sensitivity and irisin in humans, and a positive relationship between carotid intima[42]. Also,irisin could have a positive effect on inflammation. Plasma and subcutaneous adipose tissue expression of irisin are decreased in patients with obesity [43,44].

Similar to obesity, inflammation increases in the cancer process. The thermogenic protein, irisin, is also related to cancer and have an anti-inflammatory effect but its function in cancer progression is still unknown. Provatopoulou et al. showed that women with increased irisin levels had lower risk of breast cancer [45]. Another study related to breast cancer showed higher levels of irisin in women with primary breast cancers compared to spinal metastasis patients [46]. Patients with renal cell cancer had higher levels of irisin compared to the control group [47]. Bladder cancer patients had significantly lower levels of serum irisin [48]. The levels of irisin in the prostate cancer group were considerably reduced and irisin may be used as a biomarker, along with free and total PSA[49]. Research by Uğur et al. showed that irisin expression was significantly increased in oncocytic variants of thyroid cancer compared to other carcinomas of the thyroid gland [50]. Some laboratory studies indicated that irisin represses malignant breast cancer cells such as MCF-7 and MDA- MB231 proliferation, migration, and viability by exhorting caspase activity and stimulating apoptosis, but do not act on MCF-10A normal breast epithelial cells [51]. On the other hand, migration, invasion and growth of in vitro cultured pancreatic cancer cell lines (MIA PaCa-2 and Panc 03.27) were inhibited by irisin. Irisin activates AMPKα and inhibits mTOR signal pathways [52]. The same effect on motility, proliferation and viability of cells was identified for U2OS and MG-63 osteosarcoma cell lines. In the presence of irisin, the IL-6 induced epithelial–mesenchymal transition pathway is suppressed and the STAT3/Snail pathway is inhibited [53]. Irisin showed protective effect against lung cancer cells. Irisin inhibited expression of Snail in the PI3K/Akt/Snail pathway [54]. Tekin et al. showed that irisin has a cytotoxic effect on DU-145 and PC3 prostate cancer cell lines [55]. Irisin stimulated cell proliferation, migration, and invasion of hepatocellular cell lines and activated the Akt/PI3K pathway[56]. Irisin increases induced cell apoptosis of anticancer drugs like doxorubicin in pancreatic cancer cells such as MIA PaCa-2 and BxPC-3 by inhibiting the PI3K/NFkB pathway

[57]. Immunohistochemical screening of irisin in renal oncocytomas and cancers may be useful for differential diagnosis [58]. Irisin levels were significantly decreased in prostate cancer patients and irisin can be used as a biomarker, like free and total PSA [59].

Irisin demonstrated anticancer activity on lung, pancreatic, osteosarcoma, prostate and breast cancer cell lines; on the other hand, irisin did not show anticancer activity for human and mouse colon, esophageal, thyroid and endometrial cell lines and displayed no change in proliferation and adhesion properties of these cell lines [60].Unlike these, hepatocellular carcinoma cells were stimulated by irisin [56].Us Altay et al. demonstrated that FNDC5 expression was increased in white and brown adipose tissues so FNDC5, which is a precursor of irisin, may have a cachectic effect in mice with induced gastric cancer. But, higher FNDC5 expression in white and brown adipose tissue is not enough to explain the mechanism of the correlation of irisin and gastric cancer development [22]. Aydin et al. showed higher irisin expression in gastrointestinal system (GIS) tissue cancer, except liver tissues. But it is still not known why irisin does not increase in liver cancers. It is thought to be associated with gluconeogenesis [61]. It is known that there is a positive correlation between inflammation and cancer. Inflammation induces cancer growth and development. Increased irisin levels can reduce inflammatory cytokines such as TNF-a and IL-6 by inhibiting NF-kB. Thus, irisin exerts an anticancer effect by suppressing inflammation [62,63].

In the light of the above, studies conducted with the irisin molecule are still not clear. However, it is a fact that inflammation increases in many metabolic diseases such as obesity and cancer. Irisin has been shown by many studies to be an anti-inflammatory molecule. Severe inflammation associated with increased risk of mortality in Covid-19 patients. Based on this situation, a relationship between irisin and covid19 can be mentioned.

Irisin and COVID-19 Relation

SARS-CoV-2 is the third coronavirus epidemic that we have encountered after SARS-CoV and MERS-CoV infections. We have experience in the pathogenesis and immune response of coronaviruses after SARSCoV and MERS-CoV infections**.** However, studies conducted during this period show that SARS-CoV2 is highly contagious and different from our experience. The term cytokine storm has been used both in many articles and has been used for Covid-19 patients in many news and has become popular . In fact, it means that the host immune system is an uncontrolled and general creates an inflammatory response. Although the level of IL-10 detected in patients is low in SARS-CoV infections, it is also higher in SARS-CoV-2 infections. According to the current data,

patients' detected inflammatory markers (C-reactive protein, such as ferritin and D-dimer), increased neutrophil / lymphocyte ratio and some inflammatory cytokines and elevation of chemokine levels, Covid-19. It has been reported to occur with its severity and mortality.

In the studies since2012 to until today, physical activity is close related to a adipomyokine called irisin that first discovered by Boström and his friends. Nowadays some researchers have found positive correlations between irisin and physical activity [64,65] but the others have found negative relations [66,67]. Also, some authors said that irisin level can increase after acute exercise but [21,68] these increases can not be continue long-term exercise [68,69]. Some researchers have found no correlations between irisin and acute or long exercise [70,71]. It can be said that; age and BMI differencies , exercise in different ways , minutes, with or without tools are effective for getting different results in these studies. As a result acute or long term phsical activity prevents inflammation and Covid-19 risk?

The studies between irisin and Covid-19 are very limited. In a study, SARS-CoV2 effects host cells binding to angiotensin-converting enzyme-2 (ACE2), this virüs increases ACE2 and leading to inflammatuary, fibrotic activitiy and cardiovasculer damage. On the other hand, physical exercise also enhances the ACE2 expression activates PGC-1α/FNDC5/ Irisin pathway and induces cardiovasculer protection, anti-inflammatory and antifibrotic effect. In this case, after acute exercise, would increase plasma ACE2 level and therefore increase inflammation cause Covid-19? The answer should be investigated [72].

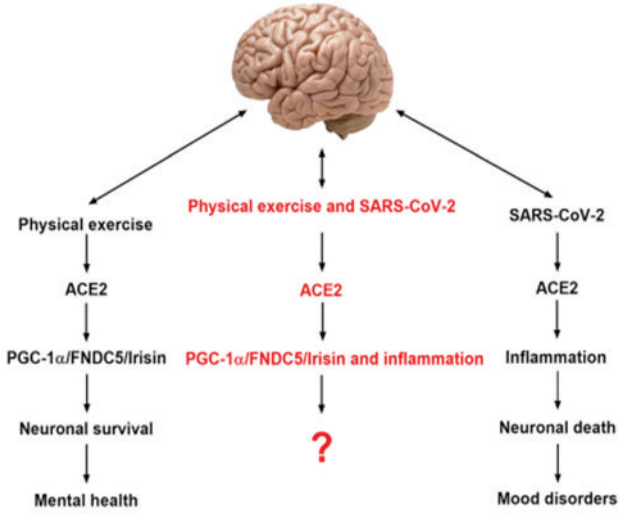

Figure 3. *Physical activity and SARS-CoV-2 have some effects on the nervous system. It is still unknown what would be the results of the regular practice of physical exercise by individuals infected with SARS-CoV-2 on central nervous system [72]*

In another study with human subcutaneous adipocyte cell culture, a positive association of irisin myokine on many genes associated with SARS-CoV-2 was revealed alsothis may apply for other tissues targeted by Covid-19.his may be promising in the treatment of Covid-19 disease, by reducing the ACE2 regulatory genes [73]. Irisin can be proposed as a new intervention to alleviate inflammation, depression and neurological signs that may arise from the Covid-19 process.[74]. Depending on the concentration of irisin treatment, the level of IL-6, TNF-α and Nuclear factor-kappa B (NF-κB), known as proinflammatory molecules, is suppressed. Also in adipoctes cultured in the presence of irisin, decreased MCP1 levels and as a result decreased migration of macrophages. [75]. So that , irisin level is associated with some anti-inflammatory markers [76]. Obesity is related with increased MCP1 and macrophages levels in adipose tissue The task of irisin is to lower the reactive oxygen species (ROS) level and increase the antioxidant parameters level thus, the beneficial effects of irisin not only associated with the regulation of genes that are related to the Covid-19, also irisin has anti-inflammatory properties related to the targeting of macrophages [73].

In summary, obesity, cancer and Covid-19 are closed related to inflammation.Most of the studies have showen that irisin is an anti-inflammatory molecule that prevents inflammation and many metabolic diseases by activating many metabolic pathways. Therefore, regular exercise increases the level of irisin and people who exercise regularly have a lower risk of developing inflammation and Covid-19? In a recent study showed that increased ACE2 level after acute exercise also increases in people with Covid-19. So, does acute exercise increase the risk of covid19? It should be investigated.In these days when Covid-19 is increasing, we should take care to stay at home as much as possible. Even so, we shouldn’t neglect physical activity even at home. Extensive studies are required to elucidate the relationship between irisin and Covid-19.

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Chapter 4

THE IMPORTANCE OF THE HIPPO PATHWAY IN DIABETES COMPLICATIONS

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Definition and Importance of Diabetes

Diabetes mellitus (DM) types 1 and 2 affect nearly 425 million people worldwide, causing severe health issues and high health costs. Furthermore, both Type 1 and Type 2 Diabetes have a poor quality of life (Arneth et al., 2019; Demir, 2021). Diabetes is a disease that arises when the pancreas fails to produce enough insulin or when the insulin that is released is ineffectively used. Insulin is a hormone that controls the body's blood sugar levels. Diabetes is characterized by hyperglycemia, which causes significant damage to others, especially nerves and blood vessels, over time (Stumvoll et al., 2005).

Diabetes is classified into two types: Type 1 and Type 2. Type 1 diabetes arises as a chronic disease involving a number of causes, including genetic predisposition, immunological dysregulation, and exposure to environmental stimuli (Acharjee et al., 2013). Type 1 diabetes mellitus (T1DM) is a chronic disease characterized by a critical reduction of insulin synthesis in the organism and a rise in blood glucose content (hyperglycemia) as a result of insulin-producing cell deficiency in the pancreas (Katsarou et al., 2017). The development of type 1 diabetes is abrupt, and beta cell mass has been reported to decrease by 70-80% at the time of diagnosis (Ryan et al., 2009). Type 1 diabetes accounts for just between 10-15% of all diabetes cases. T1DM is one of the most prevalent endocrine system disorders in children. While symptoms normally appear in infancy or adolescence, they can appear much later in life. The incidence rate varies greatly by country; it is highest in Scandinavian countries, followed by European countries, North America, and Australia. Type 1 diabetes is a rare disease in Asian countries such as China, Korea, and Japan (Katsarou et al., 2017).

Type 2 diabetes has emerged as a significant public health problem around the world. Type 2 diabetes mellitus (T2DM) is characterized by high blood glucose and lipid levels, as well as elevated carbohydrate, fat, and protein metabolism, and is classified as a metabolic syndrome. Chronic glucose and lipid elevation in the blood is caused by factors such as impaired -cell insulin synthesis function, insulin tolerance in peripheral tissues, reduced glucose utilization in peripheral tissues, and excessive hepatic glucose production (Rehman and Akash, 2017). Type 2 diabetes is the most common and accounts for at least 90% of cases. Since hyperglycemia and insulin resistance (metabolic syndrome) coexist in type 2 diabetes, the prevalence of both microvascular complications (retinopathy, nephropathy, and neuropathy) and macrovascular complications (cardiovascular diseases) is rising. Obesity, an inadequate lifestyle, and physical inactivity, for example, have been described as various pathophysiological causes responsible for compromised glucose

homeostasis in Type 2 diabetes. Type 2 diabetes appears to be on the rise, especially in low- and middle-income countries. This disease's prevalence appears to have risen dramatically, especially in Southeast Asian countries such as China and India. Statistics show that men have a significantly higher incidence of Type 2 diabetes than women (DeFronzo et al., 2015).

Importance of the Hippo Signaling Pathway

Controlling multiple biological processes holistically, such as replication, differentiation, apoptosis, and metabolism, is critical to preserving stable cell and tissue homeostasis. However, defects in these pathways are the root cause of many cancers, including cancer and diabetes, as well as many aging-related complications. Apoptosis and cell division in Drosophila melanogaster are known as a signal route because they are a powerful first editor of the Hippo signaling pathway, which controls mammalian organs and functions in both healthy and diseased tissues (Watts et al., 2017). The basic physiological role of the Hippo signaling pathway, which is characterized as maintaining metabolic homeostasis, is to restrict tissue expansion, organ size regulation, cellular proliferation, encourage apoptosis, and regeneration (Gumbiner et al., 2014; Li et al., 2017; Ardestani et al., 2018). The Hippo signaling pathway has also been shown to control cellular regeneration in tissues such as the liver, lungs, skin, and intestines (Moya and Halder, 2019). The Hippo signaling pathway in mammals is a kinase cascade composed of transcriptional co-activators, transcription factors, and their partners. Mammalian sterile 20-like protein kinases 1 and 2 (MST1/2), large tumor suppressors 1 and 2 (LATS1/2), Yes-associated protein (YAP), and PDZ-binding motif transcriptional coactivator (TAZ) comprise the Hippo signaling pathway (Wang et al., 2015; Meng et al., 2016; Ardestani et al., 2018).

When MST1/2 activation begins in the Hippo signal route, this pathway becomes active (Hippo-On). Active MST1/2 phosphorylates SAV1 and MOB1A/b, two scaffold proteins that help MST1/2 collect and phosphoryate LATS1/2 kinases in hydrophobic motifs to promote autophosphorylation and activation (Thr1079 for LATS1 and Thr1041 for LATS2). In addition to MST1/2, the MAP4K kinase family will directly phosphorylate LATS1/2. After that, active LATS1/2 phosphorylates YAP/TAZ on several residues; it has been suggested that Ser127 is a critical residue for deactivation. Phosphorylation on Ser127 I causes cytoplasmic sequestration of YAP/TAZ by exposing the docking site for 14-3-3 protein binding, and (ii) facilitates ubiquitin-proteasomal degradation of YAP/TAZ by preparing it for additional phosphorylation by other kinases. Dephosphorylated YAP/TAZ will translocate to the nucleus in the absence of activated MST1/2 and LATS1/2 (Hippo-OFF). They cannot bind

directly to DNA because they lack a DNA binding site, and instead serve as transcriptional coactivators by combining with only a few transcription factors. The TEA (TEAD) family of transcription factors has been identified as the most significant transcription factors. The development of the YAP-TEAD complex initiates the transcription of many genes that are primarily involved in the regulation of replication, survival, and differentiation. Hippo pathway, Ras-related protein family (RASSFs), kidney and brain protein (KIBRA), thousand and one amino acid (TAO1-3) kinases, MAP/microtubule affinity regulatory kinase 1, anjiyomotin family (AMOT, AMOTL1, and AMOTL2) neurons and oligodendrocytes during myelination and 2 (NF2, Merlin) (Plouffe et al., 2015; Yu et al., 2015; Meng et al., 2016; Ardestani et al., 2018).

The Hippo signaling pathway, a closely conserved organ size regulator, regulates essential cellular processes such as proliferation, viability, and differentiation, and it has been linked to a variety of pathological disorders including perturbation, cancer, and diabetes, as well as cardiovascular and neurodegenerative diseases. The Hippo signaling pathway's most significant role is to regulate cell survival and proliferation. Important evidence was discovered *in vivo* experiments examining the benefit and loss of activity of tissue-specific Hippo signaling pathway elements, as well as cell culture studies, that this pathway controls cell survival, cell cycle, and tissue regeneration, and also acts as a powerful organ size regulator (Plouffe et al., 2015; Yu et al., 2015; Meng et al., 2016; Ardestani et al., 2018). As the Hippo signaling pathway is activated (turned on), YAP and TAZ along the MST–LATS axis phosphorylate transcriptional coactivators, reducing tissue development and cell proliferation and causing them to be inactivated.

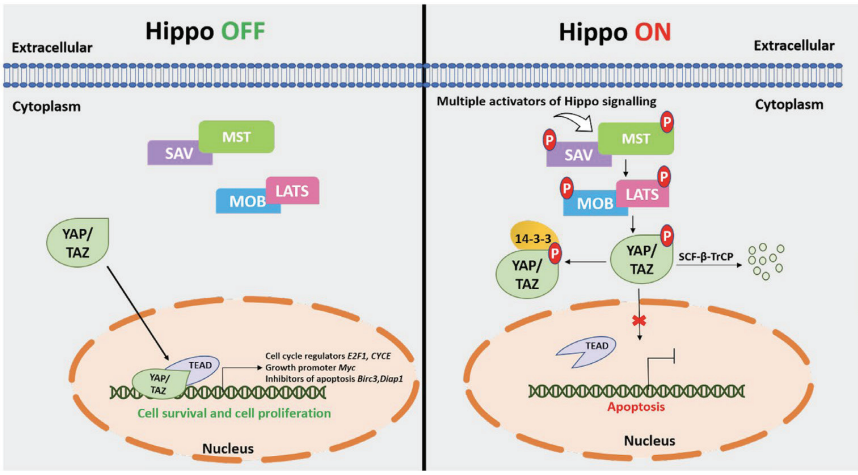

Figure 1. *The working mechanism of the Hippo pathway (Sahu and Mondal, 2020)*

YAP/TAZ terminal effectors were combined with organ growth and tissue recovery by upregulation and hyperactivation when Hippo was inactivated (closed). While the hippo signal pathway is closed, there is YAP/TAZ activation in the nucleus, and when it is open, there is YAP/TAZ suppression on the side of Hippo core components in the nucleus. It has been determined that when this signaling pathway is shut down, neoplastic development and pathological structural activations associated with various forms of cancer occur. This signaling pathway is activated (hyperactivation of MST or LATS kinases), resulting in apoptosis, which tends to be linked to childhood cardiovascular disorders, neurodegenerative diseases, and metabolic anomalies such as diabetes (Yu et al., 2015; Ardestani and Maedler, 2018; Ardestani et al., 2018; Plouffe et al., 2015; Johnson & Halder, 2014).

Many studies have been conducted on the function of this pathway, which is critical in the control of many events in the living environment, in the formulation and development of diabetes complications. The following are summaries of recent research results on the function of the Hippo signaling pathway in the development of diabetes complications.

Importance of Hippo Pathway in Cardiovascular Diseases in Diabetes

Diabetes mellitus is a significant public-health problem worldwide. One of the most common complications of diabetes is diabetic cardiomyopathy, which poses a serious danger to human health. Diabetic cardiomyopathy is a chronic complication of diabetes that manifests as a distinct unique myocardial lesion in some cardiac disorders. This disease is one of the leading causes of mortality in diabetics (Jia et al., 2016). Pathological changes in diabetic cardiomyopathy are mainly characterized by cardiomyocyte hypertrophy, apoptosis, and progressive myocardial interstitial fibrosis (Mizushige et al., 2000). Among the most serious cardiological problems affected by diabetes are myocardial fibrosis, myocardial remodeling, and cardiac failure. Myocardial fibrosis contributes to the onset and progression of diabetic cardiomyopathy and is associated with a weak prognosis of diabetic patients (Liu et al., 2017). According to some research, diabetic myocardial fibrosis is associated with oxidative stress, apoptosis, and endoplasmic reticulum stress (Varga et al., 2015; Li et al., 2016; Bugger and Abel, 2014; Nerheim et al., 2001). Apoptosis is also understood to play an important part in the formation and progression of diabetic cardiomyopathy (Zhang et al., 2017). Hyperglycemia induces myocardial tissue apoptosis through inducing oxidative stress and metabolic disorders (Liu et al., 2017). The Hippo pathway, a widespread cell signaling pathway of pathological

and physiological processes, is critical in controlling apoptosis and cell proliferation mechanisms (Pan, 2010; Radu and Chernoff, 2009). The Hippo signaling pathway has been shown in some *in vivo* experiments to play a role in the control of apoptosis in cardiovascular diseases. This pathway has also been linked to cell death by activating LATS1/2. MST1 expression, the most critical member of this pathway, has been shown to cause apoptosis in myocardial cells when upregulated or specifically overexpressed. The Hippo signaling pathway is believed to play a role in myocardial fibrosis regulating mechanisms. Overexpression of MST1 has also been linked to myocardial fibrosis (Liu et al., 2017). Activated MST induces apoptosis and inhibits cell growth and proliferation. MST1 is expressed in myocardial remodeling and cardiac dysfunction caused by stimuli such as destructive disorder, but its expression has been mediated inhibition of apoptosis in cases where tissue damage and ischemia reperfusion injury in myocardial remodeling and cardiac dysfunction caused by stimuli such as destructive disorder that may lead to improvement it has been reported (Nakamura et al., 2016; Lee et al., 2015; Hu et al., 2016; Yamamoto et al., 2003). According to recent studies, pathological activation of MST1 is closely linked to the diabetes development pathway and plays a part in the formation of myocardial fibrosis (Zhang et al., 2016; Ardestani and Maedler, 2016). In their research, Liu et al. (2017) found that the expression of proteins associated with clear myocardial fibrosis and the Hippo signaling pathway are upregulated in the cardiac tissue of diabetic rats. The Hippo signaling pathway has been shown to influence the development of myocardial fibrosis in diabetic rats. MST1/2 can phosphorylate key players in the Hippo signaling pathway, including Sav1, LATS1/2, and Mob1. Thus, MST1/2 upregulation results in apoptosis (Liu et al., 2017). In their study, Liu et al. (2017) discovered that MST1/2 and its downstream pathway proteins, MOB and LATS1/2, are distinctly upregulated in diabetes. In the case of diabetes, MST 1/2 and its downstream pathway proteins, MOB and LATS, can participate in the modulation of oxidative stress, endoplasmic reticulum stress (ERS), and apoptosis. As a result, the equilibrium between tissue injury and recovery is disrupted since the Hippo MST signaling pathway is activated in diabetes. Furthermore, since this signaling pathway is activated, it causes apoptosis and disrupts cell proliferation pathways, resulting in the development of myocardial fibrosis (Liu et al., 2017).

Importance of The Hippo Pathway in Diabetic Cardiomyopathy

Along with the exponential rise of diabetes, cardiovascular problems continue to be on the rise around the world. Diabetic cardiomyopathy

(DCM) has been identified as the primary cause of heart dysfunction disease in diabetes patients (Picano, 2003; Boudina and Abel, 2007). However, the pathogenesis and various pathways causing diabetic cardiomyopathy remain unknown, restricting the advancement of new treatment methods (Hu et al., 2018). MST1 (mammalian sterile 20-like kinase 1) is a critical part of the Hippo signaling pathway (Ardestani et al., 2018). Previous research has shown that MST1 coordinates autophagy and apoptosis in diabetic cardiomyopathy and also plays an important role in diabetic coronary microvascular malfunction disease (Zhang et al., 2016; Zhang et al., 2017; Lin et al., 2016). MST1 is thought to play a critical role in the treatment of diabetic cardiomyopathy. MST1 activity has been shown to be up-regulated in diabetic cardiomyocyte cells. This condition strongly inhibits autophagy in heart cells while also strongly promoting apoptosis, which leads to hyperglycemia-induced heart injury. MST1-enriched exosomes produced by cardiac microvascular endothelial cells in high-glucose media were found to substantially suppress glucose metabolism in cardiomyocytes by inhibiting GLUT4 membrane translocation (Hu et al., 2018). It has been discovered that MST1 protein expression improves in diabetic cardiomyocytes. In cardiomyocytes exposed to elevated glucose levels, MST1 has been shown to suppress GLUT4 membrane translocation (Hu et al., 2018). Daxx has been identified as a protein family that affects glucose uptake and plays an important role in GLUT4 translocation (Lalioti et al., 2009). It has been discovered that as MST1 expression grows, the relationship between GLUT4 and Daxx is suppressed. With decreased glucose absorption in cardiomyocytes, this syndrome leads to the production of insulin resistance. This emerging disease lays the groundwork for diabetic cardiomyopathy. Cardiomyocyte autophagy, apoptosis, and abnormal glucose metabolism all contribute to the progression of heart disease disorders. MST1 has been discovered to play a significant role in this emerging cellular phenomenon (Hu et al., 2018).

MST1 is a serine-threonine kinase that is involved in a number of biological functions such as autophagy, apoptosis, and oxidative stress (Pan, 2010; Yamamoto et al., 2003; Odashima et al., 2007). MST1 overexpression increased apoptosis in cardiac microvascular endothelial cells, while MST1 activity decreased apoptosis in cardiac microvascular endothelial cells. It was discovered that by inhibiting Silent Information regulator 1 (Sirt1) action, the negative effects of cardiac microvascular damage and cardiac dysfunction disorder caused by an increase in MST1 expression were removed. Cardiac microvascular integrity was preserved and cardiac function increased in rats lacking MST1 activity. MST1 has been shown to play a role in the pathogenesis of diabetic coronary

microvascular dysfunction disease by inhibiting the autophagy signaling pathway, increasing the activity of the apoptosis signaling pathway, and decreasing the activity of Sirt 1 in cardiac microvascular endothelial cells (Lin et al., 2016). MST1 has been shown to facilitate cardiac dysfunction disease in mice after a myocardial infarction (MI) by inhibiting autophagy (Maejima et al., 2013). Furthermore, it was discovered that upregulating the autophagy signaling pathway by inhibiting MST1 resulted in changes in cardiac dysfunction disease following infarction (Hu et al., 2016). MST 1 has been shown to specifically inhibit the function of Sirt 1. (Yuan et al., 2011). MST 1 was shown to be dense in cardiomyocytes and endothelial cells. Diabetes cardiomyopathy (DCM), characterized by the repression of the autophagy signaling pathway in heart tissue, has been linked to an improvement in the apoptosis signaling pathway. As a result of MST1's capacity to control both autophagy and apoptosis signaling pathways, it is thought to be linked to the progression of diabetic cardiomyopathy (Lin et al., 2016). MST1 phosphorylation increased in cardiac microvascular endothelial cells exposed to elevated glucose levels. Important changes in heart disease condition and microvascular disruption caused by diabetes were observed in rats where MST1 activity was removed. MST1 has been shown to suppress autophagy in cardiac microvascular endothelial cells. According to research, autophagy inhibition in cardiac microvascular endothelial cells plays a significant role in diabetes-related coronary microvascular dysfunction disease. Sirt 1 has been shown to function downstream of MST1 in the development of diabetic coronary microvascular dysfunction (Lin et al., 2016).

Diabetic cardiomyopathy is associated with decreased autophagy and increased apoptosis in the heart. MST1, or mammalian sterile 20-like kinase 1, has the ability to control both autophagy and apoptosis. When MST1 activity was inhibited in diabetic rat heart cells, autophagy function improved and cardiomyocytes were shielded from apoptosis. It was discovered that diabetic cardiomyopathy was worsened in rats where MST 1 activity was eliminated; diabetic cardiomyopathy improved, but MST1 expression was inappropriately increased. According to *in vivo* and *in vitro* results, apoptosis increased in cardiomyocytes when MST1 expression was excessively increased, but apoptosis decreased in rats when MST1 activity was removed. MST1 activity is reduced in rats, which improves autophagy functions and develops defensive properties against apoptosis, which induces diabetic cardiomyopathy (Zhang et al., 2016). The results indicate that autophagy and cardiac dysfunction co-develop and that these conditions are important in diabetic cardiomyopathy (Xie et al., 2011; He et al., 2013). In a mouse model of Type 1 diabetes, the autophagy signaling pathway was shown to be blocked in cardiomyocytes.

This inhibition has been suggested to play a role in the pathogenesis of diabetic cardiomyopathy (Zhang et al., 2016). The autophagy signaling pathway has been shown to be blocked in diabetic rats' heart tissue, while the apoptosis signaling pathway is activated. In rat myocardial tissue, an experimental model of diabetic cardiomyopathy in which MST1 activity was inhibited demonstrated enhanced glucose consumption and improved cardiac function. The findings indicated that inhibiting MST1 may be a defense measure against diabetic cardiomyopathy (Zhang et al., 2016). MST1, a Hippo signaling pathway variable, is a pro-apoptotic kinase that also regulates autophagy. The ability of MST1 to control both the autophagy and apoptosis signaling pathways has been linked to the progression of diabetic cardiomyopathy. MST 1, a pro-apoptotic signaling kinase, has been linked to the development of diabetic cardiomyopathy by inhibiting autophagy. MST1 has been discovered to control both the autophagy and apoptosis signaling pathways at the same time by inducing interdependent regulation of Beclin1, Bcl-2, and Bax (Zhang et al., 2016).

Studies indicate that inhibition of the autophagy signaling pathway leads to MST1-mediated diabetic cardiovascular pathological improvements. MST 1 regulates both autophagy and apoptosis in diabetic cardiomyopathy (Zhang et al., 2016; Del Re et al., 2014; Zhang et al., 2017). MST1 activation can cause cell death through a variety of downstream signaling pathways, including Sirtuin 1, the c-Jun N-terminal kinase pathway (JNK), caspase-3, phosphorylated histone H2B, and the Fas/FasL pathway (Wu et al., 2016).

The Importance of The Hippo Pathway in Heart Failure In Diabetes

Tissue regeneration occurrence varies greatly. Any tissues' regeneration potential is in excellent health, while others' regeneration potential is insufficient. Heart tissue is at the beginning of the list of tissues with inadequate regeneration activity. As a result, this tissue is extremely vulnerable to injury and loss of function (Monroe et al., 2019). When a human has heart disease, the procedure normally ends in death. The Hippo signaling pathway, a kinase cascade, has been shown to inhibit the proliferation and regeneration of adult heart cells, but in heart failure, this signaling pathway is up-regulated. In the experimental heart failure model, it was discovered that deletion of Salvador, one of the components of the Hippo signaling pathway, increased vascularity in the heart tissue, reduced fibrosis, and improved the heart's blood pumping function. As the Hippo signaling pathway is blocked in cardiomyocytes, the expression of stress response genes and proliferative genes is increased. It has been stated that genetic or gene therapy inhibiting the Hippo signaling pathway

could be a successful treatment strategy in ischemic heart failure by reversing organ failure. The research on this topic have yielded important results (Leach et al, 2017). Concurrent with myocardial infarction, Salvador, LATS 1 and LATS 2 deletion, which are components of the Hippo signaling pathway, is shown to cause heart function recovery (Heallen et al., 2013; Morikawa et al., 2015; Tao et al., 2016). Heart loss and ischemia reperfusion injury have been attributed to a disruption of the Hippo signaling pathway's function (Matsuda et al., 2016; Del Re et al., 2014). The Hippo signaling pathway has been shown to suppress heart cell proliferation and the function of restorative genes that enable cell survival in ischemic heart failure (Leach et al, 2017). YAP activation after a myocardial infarction has been shown to mitigate heart injury, improve cardiac function, and boost cell survival (Lin et al., 2014). Odashima et al. (2007) discovered that endogenous MST1 is essential in mediating cardiac expansion, apoptosis, fibrosis, and cardiac dysfunction disease following myocardial infarction (MI).

The Importance of Hippo Pathway in Wound Healing and Angiogenesis in Diabetes

Diabetes patients suffer from morbidity and mortality due to impaired angiogenesis and wound healing. Diabetes complications such as delayed wound healing and diabetic skin ulcers cause severe impairment and mortality in diabetic patients (Boulton et al., 2005; Brem et al., 2007). Wound healing is a multi-step mechanism that involves coagulation, inflammation, angiogenesis, tissue regeneration, and remodeling. This pathway involves stem cells, endothelial cells, fibroblasts, and keratinocytes, as well as a variety of cell proliferation, differentiation, and other functions (Martin and Parkhurst, 2004; Falanga, 2005; Gurtner et al., 2008). Abnormalities in this cell type, especially endothelial cell function, have been linked to disrupted wound healing in diabetes (Brem et al., 2007; Sawada et al., 2014; Liu et al., 2014; Qi et al., 2015).

Hyperglycemia-induced metabolic stress (Larger et al., 2004; Dobler et al., 2006; d'souza et al., 2009; Dunn et al., 2014) has been shown to impair angiogenesis and wound healing (Mehra et al., 2014). (Yuan et al., 2017). Yes-associated protein (YAP), a Hippo signaling pathway and effector, plays an important role in tissue size regulation, tissue recovery, and regeneration (Pan, 2010; Zhao et al., 2011). Mammalian Ste20-like kinases 1/2 (MST1/2), Salvadoran (SAV), and massive tumor suppressor (LATS1/2) kinases comprise the Hippo signaling pathway. The hippopotamus pathway's key downstream effectors are YAP and PDZ binding motile (TAZ) transcriptional activators. MST1/2 activates LATS1/2, then phosphorylates YAP at Ser-127 or Ser-381, resulting in

transcription of its target genes after cytoplasmic retrieval and degradation (Pan, 2010; Zhao et al., 2011; Yu et al., 2015; Varelas, 2014; Choi et al., 2015; Marti et al., 2015). YAP encourages cell growth, survival, and angiogenesis (Varelas, 2014; Choi et al., 2015; Marti et al., 2015). It prevents cell survival, replication, and angiogenesis by inactivating the Hippo signaling pathway YAP (Pan, 2010; Zhao et al., 2011; Varelas, 2014; Yu et al., 2015). The Hippo signaling pathway and YAP play critical roles in angiogenesis and wound healing (Choi et al., 2015; Marti et al., 2015; Elbediwy et al., 2016). Given the importance of the Hippo-YAP pathway in angiogenesis, it has been documented that irregularities in this pathway play a role in metabolic stress-induced inhibition of endothelial angiogenesis. While impaired angiogenesis and wound healing have been commonly identified in diabetes, the pathogenesis remains unknown (Yuan et al., 2017). The Hippo-YAP pathway is a critical regulator of tissue repair and angiogenesis (Zhao et al., 2011; Varelas, 2014; Choi et al., 2015; Marti et al., 2015; Yu et al., 2015; Hong et al., 2016). YAP stimulates angiogenesis by inducing epithelial mesenchymal transformation and expressing proangiogenic factors. To counteract YAP, the Hippo signaling pathway inhibits angiogenesis and tissue regeneration (Choi et al., 2015; Marti et al., 2015; Singh et al., 2016; Moya and Halder, 2016).

Palmitic acid-induced angiogenesis inhibition has been linked to a disruption in the function of the Hippo-YAP pathway, a key signaling factor in tissue repair and regeneration. Palmitic acid has been shown to activate MST1, inhibit YAP, and inhibit endothelial cell proliferation and tube forming (Yuan et al., 2017). It has been suggested that a disruption in the function of the Hippo-YAP pathway could be an important mechanism influencing diabetes-related disrupted angiogenesis and wound healing. Palmitic acid has been shown to stimulate mitochondrial DNA (mtDNA) release in the cytosol by stimulating the cytosolic DNA sensor cGAS–STING–IRF3 signaling, resulting in MST1 upregulation, YAP inhibition, and angiogenesis. As a result, it causes mitochondrial injury, inflammation, and endothelial dysfunction, but it can also suppress angiogenesis in endothelial cells (Yuan et al., 2017).

Protecting mitochondria from injury and/or encouraging mitochondrial elimination may be crucial in preserving endothelial angiogenesis ability (Yuan et al., 2017). Cell-cell interaction, mechanical signals, and various signaling mechanisms may all influence the Hippo-YAP pathway (Meng et al., 2016). About the fact that the PI3-kinase signaling pathway activates YAP, tumor suppressors deactivate it by activating the Hippo pathway (Fan et al., 2013; Yin et al., 2013; Matsuda et al., 2016). The cytosolic DNA sensor cGAS–STING-IRF3 signaling pathway has been shown to stimulate the Hippo signaling pathway, resulting in

YAP inhibition. The cGAS-STING-IRF3 pathway in endothelial cells has been shown to play a part in the Hippo-YAP pathway's irregularity. IRF3 inhibits angiogenesis by triggering MST1 expression and thereby inactivating YAP (Yuan et al., 2017). Palmitic acid has been shown to suppress endothelial angiogenesis by altering the Hippo-YAP signaling pathway. Palmitic acid has been shown to stimulate the cytosolic DNA sensor cGAS–STING–IRF3 pathway by causing the release of mtDNA into the cytosol. Activation of this pathway stimulates MST1 expression, which inhibits YAP. Endothelial cells undergo angiogenesis as a result of these conditions. It has been proposed that this function contributes to poor wound healing in diabetics (Yuan et al., 2017).

Importance of Hippo Pathway in Apoptosis with Beta Cell Function in Pancreas

The Hippo signaling pathway is an evolutionary conserved pathway that controls tissue growth and homeostasis in response to intracellular and extracellular signals. The Hippo pathway is significant in the pathology of many diseases, including diabetes pathophysiology. The Hippo signaling pathway has been shown to play an important role in controlling pancreatic cellular growth, as well as cell survival and physiological functions such as proliferation and regeneration. For these factors, the Hippo signaling pathway is a promising target for therapeutic agents aimed at preserving beta cell survival in diabetes (Ardestani and Maedler, 2018). Diabetes is a disease characterized by elevated blood sugar levels caused by deficiencies in insulin production or action. Currently, surveys to assess the incidence of diabetes have shown that more than 382 million individuals have diabetes. However, according to these estimates, the number of diabetics is expected to rise higher in the coming years. Diabetes, which affects many individuals globally, has grown exponentially and has become a global chronic disease, according to statistics (Vetere et al., 2014; Ahangarpour et al., 2019).

The most significant characteristic of both type 1 and type 2 diabetes is a decrease in beta cell mass or lack of function (Mathis et al., 2001; Butler et al., 2003; Vetere et al., 2014). Apoptosis, or programmed cell death, is the characteristic of reduced pancreatic beta cell mass in both type 1 and type 2 diabetes (Ardestani and Maedler, 2018). Diabetes (T2D) is a complex, multifactorial disease characterized by peripheral insulin tolerance and a substantial decline in beta cell activity and mass. Diabetes-related factors, such as glucotoxicity, lipotoxicity, and inflammation, cause endoplasmic reticulum and/or oxidative stress, resulting in compromised insulin biosynthesis and cell apoptosis (Donath et al., 2013; Alejandro et al., 2015; Huang et al., 2007; Poitout et al., 2008; Robertson et al.,

2004; Marchetti et al., 2010; Maedler et al., 2017). Disruptions in insulin signaling pathways may occur at various stages. Disruptions in regulatory pathways, on the other hand, may cause beta cell mass to decrease and inhibit their functions, resulting in their suppression by excessive activation of genes, which can lead to diabetes progression (Ardestani and Maedler, 2018).

Importance of the Hippo Pathway in Pancreatic Physiology

The Hippo signaling pathway plays an important role in pancreatic physiology. There are; The Hippo signaling pathway controls pancreatic progenitor cell proliferation, differentiation, and specification. Furthermore, this pathway controls the normal growth of the pancreas, including its size and cellular plasticity. The Hippo signaling pathway controls cell homeostasis, including beta cell activity, cell appearance, and cell proliferation. Under diabetic conditions, MST1, the central kinase of the Hippo signaling pathway, becomes active, impairing beta cell survival and function. When MST1 is blocked in *in vitro* and *in vivo* diabetes models, beta cell mass and function increase, and normal glycemic conditions are achieved. Hippo terminal effector YAP is not expressed in mature pancreatic islet cells, but when re-expressed in these cells, it promotes beta cell proliferation and survival. The Hippo signaling pathway is a diverse and complex pathway. This signaling pathway interacts with numerous other signaling pathways (intrinsic apoptotic pathways, mTOR, PI3K-AKT, and MAPK-JNK) that control beta cell survival in response to exogenous and endogenous stimuli. Targeting this signaling pathway for beta cell regeneration in diabetes will lead to the development of new treatment approaches (Ardestani and Maedler, 2018).

Several studies have shown that the Hippo signaling pathway is critical for promoting the physiological growth of the pancreas, islet cell survival, and the modulation of cell proliferation and regeneration. This signaling pathway is known to function in mammals and controls organ size (Sharma et al., 2017; Ardestani and Maedler, 2016; Ardestani and Maedler, 2018). We can see that the primary function of this signal pathway in the living system is to control cell survival and proliferation. This signaling pathway phosphorylates MST1/2, Sav1, MOB1A/B, and LATS1/2. The stimulated LATS1/2 then phosphorylates the cytoplasmic YAP / TAZ in several regions, activating this signaling pathway. When this pathway is inactive, the non-phosphorylated form of YAP/TAZ remains in the nucleus and forms a complex with TEAD transcription factors and other transcription factors, resulting in the expression of genes involved in cell survival, development, and proliferation (Boopathy and Hong, 2019). *In vivo* experiments, in addition to human cell culture studies, have shown

that pathological involvement in components of the Hippo signaling pathway (MST1/2, MOB1, LATS1/2, and Merlin) influences cell structure and functions. Furthermore, it has been discovered that an increase in YAP expression, which is a component of this signaling pathway, contributes to cell proliferation, resistance to apoptosis, and tissue development. These findings support the hypothesis that the Hippo signaling pathway acts as a powerful organ size regulator (Zhang et al., 2010, Chen et al., 2015; Song et al., 2010 Lee et al., 2010). Irregularities created by an open or closed signal path may be dangerous to one's wellbeing. Impairments in the Hippo signaling pathway are linked to unregulated cell growth and cancer (Harvey et al., 2013; Yu et al., 2015; Plouffe et al., 2015), while deficiencies in open regulation contribute to specific cell losses, resulting in cardiomyopathy and diabetes (Harvey et al., 2013; Yu et al., 2015; Plouffe et al., 2015). (Ardestani et al., 2014; Shao et al., 2014; Liu et al., 2017; Hu et al., 2018). The primary evidence for using this signaling pathway for clinical purposes in diabetes derives from observations of the influence of this pathway on the survival, proliferation, and insulin metabolism of insulin-producing cells (Ardestani et al., 2014). Diabetes, MST1 hyperactivation, cardiomyopathy (Zhang et al., 2016; Lin et al., 2016; Hu et al., 2016; Yang et al., 2018), nephropathy (Wu et al., 2016; Yang et al., 2020), cardiovascular diseases (Odashima et al., 2007; Hu et al., 2017; Hu et al., 2018), neurodegenerative diseases ((Salojin et al., 2014; Wang et al., 2020). In experiments on the systemic deletion or inhibition of MST1 in mice, positive findings for the treatment of various pathological conditions were obtained (Salojin et al., 2014; Ardestani et al., 2014; Lin et al., 2016; Wang et al., 2017 ; Hu et al., 2016).

Given the importance of the Hippo signaling pathway in cell proliferation, differentiation, and regeneration, it is not shocking that it plays a role in pancreatic structural and functional growth (Faizah et al., 2020). According to research, the Hippo signaling pathway can control pancreatic production and cell specialization in humans and mice at an early stage of embryonic development (Mo et al., 2014; Sharma et al., 2017). The Hippo signaling pathway, which plays an important role in tissue homeostasis, tends to play an important role in preserving the normal activity of the cells that make up the tissue, as well as in controlling the healing process after tissue injury. *In vivo* studies have revealed that this signaling pathway is critical in cell regeneration, particularly in liver, intestine, and heart cells after tissue damage (Fan et al., 2016; Loforese et al., 2017; Heallen et al., 2013; Hong et al., 2016).

Latest research has shown that the Hippo signaling pathway can be used as a therapeutic target in the treatment of diseases. With findings indicating that the Hippo signaling pathway regulates beta cell

survival, cell proliferation, and insulin metabolism, this pathway tends to provide good cues for the advancement of novel treatment methods for therapeutic purposes in diabetes (Ardestani and Maedler, 2018; Ardestani et al., 2014; Ardestani et al., 2019). Abnormal MST1 activity is not limited to diabetic beta cells; abnormal MST1 activity has been linked to cardiomyopathy, nephropathy, coronary disorders, diabetes complications, neurodegenerative diseases, and autoimmune system diseases. Furthermore, studies show that when MST1 activity is inhibited or deleted, positive findings for the treatment of various pathological conditions are obtained (Ardestani and Maedler, 2018; Zheng and Pan, 2019; Ansari et al., 2019; Yeung et al., 2019; Wang et al., 2017; Li et al., 2017; Hong et al., 2016).

Role of Hippo Signaling in Pancreatic Biology

MST1 has been discovered to play a significant role in the apoptosis of mouse and human pancreatic islet cells (Ardestani et al., 2014; Sharma et al., 2017). YAP1 has been shown to be necessary for pancreatic progenitor cell proliferation (Zhang et al., 2013). It has been discovered that when the pancreas' endocrine role is disrupted, beta cell mass increases as YAP expression in the pancreas is reactivated via the vector (George et al., 2015). MST1/2 has been shown to control essential cellular functions such as cell proliferation, differentiation, and apoptosis as part of the Hippo signaling pathway. It is also well understood that oxidative stress increases MST1/2 function (Qin et al., 2013). Direct phosphorylation of MST1 kinase under stress conditions activates forkhead box proteins (FOXO), which causes apoptosis (Valis et al., 2011). MST 1 has been shown to phosphorylate FOXO transcription factors, thus activating them. In mammalian neurons, this activity has been shown to mediate cell death (Yuan et al., 2009). MST1 has been identified as a crucial mediator of apoptotic signaling that results in beta cell death by activating pro-apoptotic kinases. Overactivation of MST1 in beta cells in diabetogenic conditions contributes to a significant increase in mitochondria-dependent apoptotic cell death in human and rodent beta cells through upregulation of BIM. Apoptosis was weakened and beta cell regeneration was improved in MST1 knockout mice treated with various low-dose streptozotocin treatments. As a result, it was discovered that hyperglycemic symptoms improved. Furthermore, it was discovered that under these conditions, beta cell mass improved due to increases in beta cell survival and proliferation. According to the data, apoptotic cell death increases in beta cells due to increased MST1 activity, and insulin metabolism is inhibited by various mechanisms (Ardestani et al., 2014). YAP/TAZ, a member of the Hippo signaling pathway, has been shown in studies to facilitate the

differentiation and proliferation of progenitor cells in a variety of organs. Furthermore, cytoplasmic sequestration of YAP/TAZ has been found to be required for tissue homeostasis (Yu and Guan, 2013). A growing body of evidence indicates that the Hippo signaling pathway is significant in cardiac anatomy and physiology (Windmueller and Morrisey, 2015). YAP has been discovered to play a regulatory function in cardiomyocyte proliferation and embryonic heart size maintenance (Xin et al., 2011). It was discovered in the experimental model that YAP activation in heart tissue after myocardial injury restored cardiac functions and enhanced the risk of survival (Lin et al., 2014).

Role of Hippo Signaling Pathway in Diabetic Retinopathy

One of the most serious complications of diabetes is diabetic retinopathy (DR). It is now one of the leading causes of blindness. Retinopathy is described by vascular irregularities, such as increased permeability of the eye's arteries and the development of new blood vessels on the retina (Cheung et al., 2010; Lechner et al., 2017). The Hippo signaling pathway's most significant role in the organism is to regulate the maintenance of its activity in a stable manner by regulating tissue size. This pathway controls tissue and organ size by controlling cell formation, proliferation, differentiation, and apoptosis (Meng et al., 2016; Ma et al., 2019). Kinase proteins such as mammalian sterile 20-like protein (MST), massive tumor suppressor protein (LATS), MOB kinase activator 1 protein (MOB1), and Salvador I protein are core members of this pathway (HSAV1). By activating the YAP and the transcriptional co-activator PDZ binding motif (TAZ) protein, this kinase cascade phosphorylates the TEAD family of transcription factors (Boggiano et al., 2011). Lats (Dai et al., 2013) and YAP (Tsuneki and Madri, 2014) have been shown to affect endothelial and vascular smooth muscle cell proliferation, respectively (Xie et al., 2012; Wang et al., 2012). It has been discovered that YAP, the effector protein of the Hippo signaling pathway, is expressed in retinal vessels and is also implicated in endothelial sprouting and angiogenesis pathways (Choi et al., 2015). (Hao et al., 2017). It has been discovered that mammalian Müller glial cells lack proliferative and regenerative capacities. The Hippo signaling pathway has been shown to inhibit the proliferation and reprogramming of normally mammalian Müller glial cells. However, inhibition of the Hippo signaling pathway in Müller glial cells in mammals has been shown to activate these cells' regenerative potential (Rueda et al., 2019; Hamon et al., 2019). The operation of the Hippo signaling pathway closely regulates the physiological development of the eye. Defects in the functioning of this signaling pathway, however, have been shown to induce severe eye lesions by inducing defects in

the growth and development of optic tissues (Moon and Kim, 2018). According to the findings, precise control of the Hippo signaling pathway may be critical in maintaining eye health and preventing eye diseases (Pfleger, 2017). Diabetic retinopathy is characterized by angiogenesis, which occurs in tandem with endothelial cell proliferation. As a result, it has been suggested that the Hippo signaling pathway is essential in diabetic retinopathy. The function of the Hippo signaling pathway has been shown to be disrupted in diabetic rat retinal tissue (Hao et al., 2017).

The Hippo signaling pathway has been shown to be active in cell proliferation and angiogenesis by regulating vascular endothelial growth factor (VEGF). While not all participants in the cross-talk between the Hippo pathway and VEGF are fully understood, it has been stated that it can function as an ERK signaling pathway mediator (Zhang et al., 2009). It has been discovered that YAP, a member of the Hippo signaling pathway, phosphorylates the ERK signaling pathway. It has been shown that an active ERK signaling pathway increases VEGF expression (Essafi-Benkhadir et al., 2010; Curry et al., 2008; Hao et al., 2017). As a result, it has been proposed that the Hippo signaling pathway stimulates the ERK signaling pathway, resulting in angiogenesis by modulating VEGF in this signaling pathway. The functions of all members of the Hippo signaling pathway, including MST, LATS, YAP, TAZ, and TEAD, were observed to alter in the retinas of diabetic retinopathy rats. As a result, these findings indicated that the Hippo signaling pathway could play a role in the development of diabetic retinopathy. In diabetic rats, the activation of the ERK signaling pathway was found to be increased with VEGF protein expression. It has been reported that the VEGF protein Hippo signaling pathway through ERK signaling may be downstream targets in diabetic retinopathy (Hao et al., 2017). Upstream regulators of the Hippo signaling pathway, GPCR, SCRIB, and its fate, have been shown to control vascular cell proliferation, cell migration, and insulin secretion (Harvey et al., 2013; Michaelis et al., 2013; O'Hayre et al., 2014; Yamagata et al., 2002). As a result, the Hippo signaling pathway's function is intimately related to angiogenesis and glucose metabolism. Diabetic retinopathy is distinguished by angiogenesis and hyperglycemia. As a result, defects in the function of the Hippo signaling pathway have been proposed as one of the molecular pathways causing diabetic retinopathy. It has been claimed that the Hippo signaling pathway is essential in diabetic retinopathy and could be a possible drug goal for diabetic retinopathy treatment (Hao et al., 2017).

Table 1. *List of key Hippo pathway genes in fruit fly (Drosophila melanogaster) and the respective orthologs in humans (Boopathy and Hong, 2019)*

Human protein name	**Human *gene name***	***Drosophila* protein (*gene name*)**
Mammalian STE20-like kinase 1 (MST1)	STK4	Hippo *(Hpo)*
Mammalian STE20-like kinase 2 (MST2)	STK3	
Neurofibromin-2/Merlin	NF2	Merlin *(Mer)*
MOB kinase activator 1A	MOB1A	Mats *(Mats)*
MOB Kinase Activator 1B	MOB1B	
Salvador homolog 1	SAV1	Salvador *(Sav)*
Large tumor suppressor kinase 1	LATS1	Warts *(Wts)*
Large tumor suppressor kinase 2	LATS2	
Yes-associated protein 1/ YAP	YAP1	Yorkie *(Yki)*
WW Domain Containing Transcription Regulator 1/TAZ	WWTR1	
TEA domain family member 1	TEAD1	Scalloped *(Sd)*
TEA domain family member 2	TEAD2	
TEA domain family member 3	TEAD3	
TEA domain family member 4	TEAD4	
Kidney and brain expressed protein (Kibra)	WWC1	Kibra *(Kibra)*
Crumbs homolog 1 (Crumbs)	CRB1	Crumbs *(Crb)*

Role of The Hippo Signaling Pathway in Diabetic Nephropathy

Diabetes symptoms affect almost all body tissues, including the skin, nervous system, liver, and cardiovascular system. Renal tissue is one of the most severely damaged. Diabetic nephropathy (DN) is the leading cause of end-stage renal disease and is one of the most frequent microvascular complications (Cooper, 1998; Qi et al., 2017). Diabetic nephropathy is characterized by glomerular mesangial cell proliferation, mesangial matrix enlargement, and basement membrane thickening. This evolving scenario eventually leads to glomerular sclerosis (Kanwar et al., 2008; Qian et al., 2021). Proliferation of glomerular mesangial cells is seen in this image during the early stages of diabetic nephropathy. With glomerular mesangial cell proliferation, a series of improvements arise in the physiological activity of cells. Both modifications result in an increase in the mesangial cell matrix as well as an increase in the production and aggregation of extracellular matrix components including collagen IV and laminin, all of which are essential factors in the development of glomerular sclerosis (Wang et al., 2017).

This structural changes in kidney tissue are normal and influential pathological changes in diabetic nephropathy (Lei et al., 2019). A protein kinase signaling pathway, the Hippo signaling pathway. It has been discovered in recent years to play a role in the modulation of cell formation, proliferation, and apoptosis by controlling the expression of downstream genes involved in organ production, tissue replication, embryonic development, and tumor development (Yin and Zhang, 2011; Yu and Guan, 2013; Irvine, 2012; Harvey et al., 2013; Ardestani and Maedler, 2018). The Hippo signaling pathway is made up of three interconnected modules: central protein kinase, downstream transcription, and upstream regulatory (Chan et al., 2011; Ardestani et al., 2018; Ardestani and Maedler, 2018; Dong et al., 2007; Zhao et al., 2008). YAP/TAZ do not have distinct DNA binding regions, but they interact with transcription factors in DNA to bind to target gene promoters. YAP/TAZ binds to TEAD 1-4 transcription factors to regulate genes that control cell proliferation and death (Boggiano et al., 2011; Yu et al., 2013). When the Hippo signaling pathway is blocked, YAP enters the nucleus and interacts with the transcription factor TEAD to promote the expression of target genes, controlling the cell cycle and DNA synthesis and maintaining cell replication (Harvey and Tapon, 2007; Sahu and Mondal, 2020). It has been established that the Hippo signaling pathway regulates the proliferation of renal tubular epithelial cells (Kai et al., 2016). It has been established that the Hippo signaling pathway plays a role in the proliferation of glomerular mesangial cells exposed to elevated glucose levels (Lei et al., 2019).

The Hippo signaling pathway was discovered to be inactive in glomerular mesangial cells subject to elevated glucose levels as well as diabetic mice. When YAP expression rises, it binds to TEAD, causing glomerular mesangial cell proliferation. Furthermore, reactivating the Hippo pathway through transfection in a high glucose environment inhibits both glomerular mesangial cell proliferation and extracellular matrix increase (Lei et al., 2019). Previous research has established the importance of the Hippo signaling pathway in glomerular mesangial cell proliferation caused by high glucose levels. It has been documented that YAP is upregulated in response to diabetes, resulting in increased expression of profibrotic factor connective tissue growth factor. Silencing of YAP gene expression has been shown in cell culture experiments (LLCPK-Cl4 cells) to greatly inhibit high glucose-induced cell proliferation (Lei et al., 2019; Chen and Harris, 2016). The proliferation of glomerular mesangial cells exposed to elevated glucose levels has been shown to increase. This rise has been linked to the inactivation of the Hippo signaling pathway. However, it has been noted that if this mechanism is not stopped, kidney functions are compromised (Lei et al.,

2019). TAZ expression, a downstream effector of the Hippo signaling pathway, was shown to be downregulated in proximal tubule cells in Type 1 and Type 2 diabetes experimental models. Although it has been reported that activation of the EGF receptor (EGFR) or Hippo signaling pathway is associated with cell proliferation, differentiation, and death, dysregulation in the action of these signaling pathways is associated with tumor formation. According to research, an increase in EGFR activation in renal epithelial cells aggravates diabetes-induced kidney damage. For the first time, researchers discovered cross-talk between the EGFR signaling pathway and the Hippo pathway in the diabetic kidney. It has been proposed that EGFR-mediated YAP signaling is a key mechanism in the production and progression of diabetic nephropathy (Chen and Harris, 2016). The Yes-associated protein (YAP) is an essential component of the Hippo signaling pathway. This protein has been shown to control organ size, cell proliferation, differentiation, and apoptosis cycles, as well as to inhibit epithelial-mesenchymal transformation and cell interaction. It has been suggested that YAP plays a significant role in diabetes-related kidney injury. Furthermore, based on the findings, it has been proposed that there could be a connection between the inhibition of YAP activity and the ability to delay the progression of diabetic nephropathy (Ma et al., 2019).

Diabetes has been shown to suppress the Hippo signaling pathway when activating YAP. According to reports, this condition promotes the proliferation of mesangial cells. It has been discovered that increasing the activation of the PI3K/Akt signaling pathway promotes cell proliferation by inhibiting the Hippo pathway. Both symptoms have been linked to the progression of diabetic nephropathy over time (Qian et al., 2021).

MST1, a mammalian serine/threonine-protein kinase that is an integral component of the Hippo pathway involved in cellular proliferation and differentiation, has been linked to the pathogenesis of metabolic disorders, kidney diseases, and cancer. MST1 is inactivated in the cytoplasm under normal conditions (Wu et al., 2016). MST1 activation has been shown to be greatly decreased in diabetic nephropathy. *In vivo* studies have shown a correlation between MST1 deficiency and renal dysfunction and fibrosis development. It has also been discovered that overexpression of MST1 successfully corrects diabetic nephropathy-induced kidney fibrosis. When MST1 activity is blocked, the YAP protein binds to the TEAD protein, resulting in the formation of a YAP-TEAD heterodimer. This formation has been discovered to upregulate TEAD activation, encouraging epithelial mesenchymal transformation (EMT) in renal tubular epithelial cells and inducing renal fibrosis in diabetic nephropathy (Yang et al., 2020). In the MST1 knockdown model, compromised kidney function was greatly increased, and proteinuria levels were reduced (Wu et al., 2016).

In addition to metabolic disorders (diabetes, obesity, and nonalcoholic fatty liver disease), new evidence indicates that MST1 plays a critical function in kidney diseases. Deregulation of the Hippo-TAZ pathway was discovered in a chronic kidney disease study (Anorga et al., 2018).

In an animal model study and cell culture (HK-2) study, it was discovered that inactivated MST1 induced renal fibrosis in diabetic nephropathy by facilitating epithelial-mesenchymal transformation in renal tubular cells. MST1 has been shown to be a central player in the development of fibrosis and to contribute to the growth of fibrosis in tissues such as the heart, kidney, lung, and liver. By activating YAP, MST1 inactivation in tubular cells allows it to pass towards the cell nucleus. In the cell nucleus, a YAP/TEAD complex is formed. This combination results in tubular epithelial cell fibrosis and epithelial mesenchymal transformation. The development of the YAP-TEAD complex is greatly reduced when MST1 expression is increased in diabetes. This reduction has been shown to avoid the production of renal fibrosis in diabetic nephropathy in *db/db* mice by preventing epithelial-mesenchymal change (Yang et al., 2020). According to research, MST1 is linked to diabetic cardiomyopathy (Hu et al., 2018). Furthermore, it has been discovered that MST1 levels are substantially higher in primary rat podocytes cultured under hyperglycemic conditions (Wu et al., 2016). It has been established that in rats with diabetic nephropathy, MST1 inactivation can result in the development of fibrosis. In diabetes, increased MST1 expression can prevent renal fibrosis (Yang et al., 2020).

YAP has been shown in studies to play responsive and complex roles in a variety of metabolic disorders, including obesity, Type 2 diabetes, and non-alcoholic fatty liver disease (NAFLD) (Koo and Guan, 2018). Diabetes has been linked to an increase in YAP and TEAD expression in kidney tissue. It has been proposed that there is a link between increased YAP expression and diabetic nephropathy injury. It has also been stated that activated YAP is linked to diabetic nephropathy clinical manifestations (Ma et al., 2019). High glucose was discovered to be an effective inducer of YAP expression in the cell culture sample (HK-2) (Yang et al., 2020). MST1 levels were shown to be substantially higher in primary rat podocyte cells cultured under hyperglycemic conditions in an *in vitro* trial (Wu et al., 2016). MST1 activation has been shown to induce fibrosis in diabetic nephropathy by inducing dysregulation in proteins associated with epithelial mesenchymal passage (Yang et al., 2020).

There is growing evidence that it acts as a physiological regulator of YAP in podocyte cell apoptosis (Campbell et al., 2013; Schwartzman et al., 2016; Huang et al., 2016). Apoptosis is increased in podocyte cells when YAP expression is suppressed in a high-glucose environment, but

when YAP expression is increased, apoptosis is greatly reduced (Huang et al., 2018).

Importance of Hippo Signaling Pathway in Cognitive Dysfunction in Diabetes

The most prevalent form of dementia is Alzheimer's disease. It affects tens of millions of people worldwide, and the number is growing rapidly. This disease has a significant social and economic effect. It has been determined that amyloid β (Aβ) protein misfolding and intracellular aggregation of misfolded tau protein in neurofibrillary tangles induce memory loss and confusion in the human. According to reports, this situation can result in cognitive impairment over time (Chen et al., 2017). Alzheimer's disease is scientifically distinguished by a progressive and steady decline in cognitive ability, as well as specific neuronal and synapse loss (Murphy and LeVine, 2010). MST1 hyperactivation has been shown to have an important effect on amyloid β (Aβ) proteolytic regulation (Jang et al., 2007; Huang et al., 2012). As a result, it has been proposed that there is a connection between Hippo signaling pathway overactivation and the development of neurodegenerative diseases (Plouffe et al., 2015). According to new research, the Hippo signaling pathway has a dual effect on glucose metabolism (Wang et al., 2015; Peng et al., 2017). It has been documented that the Hippo signaling pathway plays an important role in the formation and progression of diabetes-related cognitive dysfunction. Hippo signaling in the brain and peripheral tissues is manifested in a proportionally different manner in diabetes-related cognitive dysfunction (MST1 or p-YAP/YAP ratio). Furthermore, it has been discovered that the amount of LATS1 in the liver, kidney, skeletal muscle, and intestinal tissues is reduced in diabetes (Yu et al., 2019). MST1, a key kinase in the Hippo signaling pathway that induces apoptosis, has been linked to neuronal death in studies (Li et al., 2018a, b). The downstream mediator of the Hippo signaling pathway, YAP, has been shown to be abundant in human brain tumors and to promote glioblastoma formation. As a result, YAP has been discovered to play a critical role in natural brain growth (Orr et al., 2011). MST1 and p-YAP/YAP levels, which are components of the Hippo signaling pathway in various brain regions of diabetic rats, were shown to be increased in rats with cognitive dysfunction. Finally, it implies that changes in the activation of the Hippo signaling pathway in the brain and peripheral tissues can lead to diabetes-induced cognitive dysfunction (Yu et al., 2019).

Conclusion

In vivo and *in vitro* experiments performed during the last few years have yielded important knowledge regarding the role of the Hippo signaling pathway. According to these findings, the Hippo signaling

pathway is not only a tissue and organ growth regulator, but it also plays an important role in controlling the function and functioning of the whole organism. According to research, this signaling pathway influences tissue repair and regeneration mechanisms, as well as energy metabolism. The deficiency in the function of the Hippo signaling pathway has been linked to the formation and progression of many diseases. It has been established that the components of this signaling pathway play a role in the pathogenesis of various diseases. As a result, identifying molecules that can modulate the components of the Hippo signaling pathway and elucidating the mechanisms of action of these molecules would be extremely beneficial in the creation of novel treatment methods for human health today. Furthermore, determining the impact of this signal pathway on other organ functions arises as a significant research subject. With thorough investigation of its impact on immunology, stem cell biology, metabolism, and processes, it is shown that this signaling pathway has the capacity to reach valuable knowledge about the proper functioning of the organism. Furthermore, it is shown that evaluating the association of this signaling pathway with other signaling pathways will provide us with valuable knowledge regarding disease progression, treatment strategy growth, and metabolic function.

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Chapter 5

DIGITAL COMPLETE DENTURES

Eyyüp ALTINTAŞ[1]

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Design and production technology with computer (Computer Aided Design / Computer Aided Manufacturing, CAD / CAM) through advanced data collection and production capability has a wide range of use in dentistry. CAD / CAM technology is routinely applied in the preparation of inlay and onlays, full crowns, fixed and removable partial dentures, implant supports, maxillofacial prostheses, infrastructures of removable and fixed implant-supported prostheses(Miyazaki, Hotta, Kunii, Kuriyama, Tamaki, 2009:44-56); (Bidra, Taylor, Agar, 2013:361-6).

In recent years, CAD / CAM technology has been applied in total prosthesis production, appears as an alternative to conventional methods. To create anatomical details and profile computer such as computed tomography (CT), magnetic resonance imaging(MRI) assisted medical imaging methods and laser surface scanners and optical systems is used and in this way, it is possible to prepare prostheses with contours and tissue adaptation are at a good level(Bidra et al.,2013:361-6); (Maeda, Minoura, Tsutsumi, Okada, Nokubi, 1994:17-21); (Kawahata , Ono, Nishi, Hamano, Nagaoka, 1997:540-8); (Kanazawa, Inokoshi, Minakuchi, Ohbayashi, 2011:93-6); (Goodacre, Garbacea, Naylor, Daher, Marchack, Lowry, 2012:34-46).

Clinical and laboratory protocols of commercial manufacturing companies, involves manual and digital combined use of procedures. It is aimed to deliver the total prosthesis to the patient at the only 2 clinics appointment with also can be milled during the production phase engraving suitable prepolymerized polymethylmethacrylate (PMMA) blocks or using the layering method from rapid prototyping techniques(Bidra et al.,2013:361-6); (Kawahata et al., 1997:540-8); (Goodacre et al., 2012:34-46).

The distinctive feature of rapid prototyping techniques is that to produce in CAD / CAM systems not by removing and processing the material from the main source as it is, adhering layers (layer by layer) with the help of technologies such as laser and numeric control. Through the layering technique, the internal details of complex bodies and undercut areas are created smoothly. Total prostheses preparation with CAD / CAM technology considered more appropriate than preparation with prototyping techniques for clinic use. Prepolymerized acrylic blocks are less porous and produced under high temperature and pressure than conventional acrylic resins produced for use with this technology, Which asserted they are more resistant and have low surface roughness and homogeneity(Bidra et al.,2013:361-6); (McLaughlin, Ramos, 2015:493-7).

Development of CAD / CAM Technology in Total Prosthetics

In 1994, by a group of Japanese researchers including Maeda et al. published the first report noticing the production of total prostheses with computer-aided rapid prototyping technology from photopolymerizing resin. These researchers have realized production of total prosthesis using the 3-dimensional (3D) lithography technique, by attaching artificial teeth are applied to the tooth-colored composite resin to bases produced from photopolymerizing resin after scanning the silicone impression with 3D laser scanners and cameras. The outer shells were manufactured by using laser lithography, and the inside was filled with acrylic composite resin(Maeda, et al., 1994:17-21).

A few years later, In 1997, Kawahata et al. have explained the concept of the preparation of a duplicate of the prosthesis from modeling wax at the CNC (Computer Numerical Control) milling device after scanned the patient's existing prosthesis, copied as digitally(Kawahata et al., 1997:540-8).

Busch and Kordass, firstly in 2006 worked on feasibility of with special computer software and artificial tooth arrangement using the anatomical measurements and averages made on scanned toothless models(Busch, Kordass, 2006:113-20).

On the other hand, Sun et al. (2009); have designed a technique in which it is used scanning toothless models and occlusal templates. By developing a new program in a direction that will allow it to be made virtual tooth arrangement and prepared flask. Virtual flasks were built according to the CAD denture casts, and individualized bodily flasks were made with a 3D printer and the artificial teeth are placed in their own places in the printed flask then the prosthesis was finished in a conventional way(Sun, Lü, Wang, 2009:266-72).

Wu et al., In 2010, reported that thanks to the combined use of the laser rapid prototyping with CAD / CAD technology, titanium metal bases are also can be produced for total prostheses(Wu, Gao, Tan, Chen, Tang, Tsui, 2010:309–15).

In the following years, many researchers development of this field with their studies by digitally prepared tooth arrangement, the use of Conical Beam Computed Tomography (KIBT) in the scanning of total prostheses and CNC milling and rapid prototyping technologies in the production phase(Bidra et al.,2013:361-6); (Kanazawa, et al., 2011:93-6); (Goodacre et al., 2012:34-46); (Inokoshi, Kanazawa, Minakuchi, 2012:40-6).

Among these researchers, Kanazawa et al. (2011), recorded the mucosal surfaces and jaw relations with 3D KIBT by scanning with the patient's existing total prosthesis. Then virtual prostheses created using 3D CAD software, manufactured in CNC device by milling and artificial teeth connected to this base(Kanazawa, et al., 2011:93-6).

Goodacre et al. (2012), have realized the first trial on the patient with prepared by milling from prepolymerized blocks and which the artificial teeth can be attached to CAD / CAM prosthesis base(Goodacre et al., 2012:34-46).

Although techniques used for the preparation of CAD / CAM prostheses are very promising, they require the stage of obtaining a measure and model(Arslan, 2016:4).

According to the results of the suitability of using intraoral scanners as an alternative to the conventional impression technique of toothless jaws in-vitro study in which it was evaluated by Patzelt et al. (2013); it has been determined that the commercially available intraoral cameras are not sufficient for in vivo use(Patzelt,Vonau, Stampf, Att, 2013:914-20).

With the existing systems, total prostheses can be delivered to the patient at only 2 clinics appointment(Infante, Yilmaz, McGlumphy, Finger, 2014:351-5).

However, occlusal vertical dimensions, lip support, maxillomandibular relations, incisal edge position and checking the mandibular occlusal plane to verify on the patient is not possible exist. In order to overcome this disadvantage, thought to be created only the prosthesis bases, which are recorded and will be set of tooth instead the entire prosthesis(McLaughlin et al., 2015:493-7).

The use of new technologies and materials in the production of total prostheses is the most one of it's important aims is to reduce polymerization shrinkage. Polymerization shrinkage is eliminated in prosthesis bases produced with the CAD / CAM technology, and prostheses with better tissue compatibility with less occlusal errors can be prepared(McLaughlin et al., 2015:493-7).

Available CAD / CAM Total Prosthetic Systems

Commercial manufacturing companies (Dentca, Avadent, Baltic etc. System) clinical and laboratory protocols contains the combined use of manual and digital procedures and it is aimed that the total prosthesis can be delivered to the patient in just 2 clinical appointments with CAD / CAM or rapid prototyping technology(Arslan, 2016:13).

Since it is not possible to recording dynamic muscle and jaw movements with intraoral 3D scanners and made full digital impression of edentulous arcs, made manually with conventional techniques and materials. Other manual stage is artificial teeth attached to the sockets created on the prosthesis base in the laboratory(Arslan, 2016:13).

First clinical appointment collect systematic data such as the impression, occlusal vertical dimension, maxillomandibular relations and tooth selection, the second clinical appointment is to includes adaptation of the prosthesis and process of delivering to the patient(Arslan, 2016:13).

AvaDent™ Digital Prostheses (Global Dental Science Europe BV, Tilburg, Netherlands) and Baltic Prosthetic systems (Merz Dental GmbH, Lütjenburg, Germany) uses milling prostheses from prepolymerized acrylic resin blocks and the technique of connecting artificial teeth to this base in production phase. With these systems, can be prepared upper and lower jaw total prostheses, implant supported overdentures, immediate total prostheses, hybrid prostheses and guides for implant surgery. Currently, it is also possible to prepare one-piece monolithic prosthesis which to milled artificial teeth together with the base(Bidra, Farrell, Burnham, Dhingra, Taylor, Kuo, 2016:578-86).

In Dentca™ (Dentca Inc., Los Angels, USA) system is prepared trial prosthesis with rapid prototyping technique, definitive prosthesis is composed conventional method in created by the 3D printers flasks. With this technique, it is possible to prepare prostheses only the lower and upper jaw(Arslan, 2016:13).

There are disadvantages with these systems such as occlusal vertical dimension, maxillomandibular relations, lip support, inability to fully assess maxillary incisors edge position, mandibular occlusal plane cannot be detected, the material used and the laboratory as higher cost than conventional technique. Also it is generally not possible to try the prosthesis intraorally before completion constitutes a significant disadvantage for the physician and the patient(Arslan, 2016:14).

Preparation stages of digital total prostheses

1) Impression Stage: Patient's own existing prosthesis can be used for made impression and interocclusal records by duplicating individual impression tray is used for impression and occlusal recordings

2) Centric relation record is saved with the gothic arc tracing

3) With the help of guides to be used by determining the patient's midline and lip support, the size of the teeth to be used is determined.

4) The records and impressions made from the patient in the clinic are scanned with laser and recorded digitally.

5) Using a computer software program;

a- Maxillomandibular occlusal relationship records are digitally combined with the scanned impression record

b- Prosthesis borders are determined and marked

c- Tissue surfaces of the denture base are created.

d- Virtual tooth arrangement is done.

e- The final form of the prosthesis is created.

6) Prosthesis is produced by milling from prepolymerized blocks(Infante et al., 2014:351-5).

Production Techniques of CAD / CAM Complete Dentures

1) AVADENT™ /DentsplySirona technique

The AvaDent system helps manufacture two types of prostheses using subtractive manufacturing(Kanakaraj, Kumar, Ravichandran, 2021:492). The Avadent system enables the production of complete dentures, record bases, single arch full dentures, immediate full dentures, temporary dentures, locked occlusal splints, radiographic guides, verification apparatus, bone reduction guides, conversion prostheses, obturators and finished hybrid prostheses(Baba, 2016:203–208).

1) Milled base where teeth and base are one unit (AvaDent XCL)

- Single layer tooth with XCL-1 dentine core

- Multilayered tooth with XCL-2 natural form of dentine and enamel core

2) Consisting of milled prosthetic base and glued teeth(Kanakaraj, et al., 2021:492).

First Appointment: In this appointment, different techniques can be used for jaw relationship records and definitive impression making(Baba, 2016:203–208).

- For impression making and recording of interocclusal relationship, existing prostheses are used as trays after duplication.

- Thermoplastic toothed prostheses in stock are used as impression trays for interocclusal registration with impression making.

- The maxillary and mandibular impressions are made separately by using the traditional way of elastomeric impression material that is not juicy.
- Clinical recordings are taken using the Anatomical Measurement Device (AMD) with maxillary and mandibular partial arch trays(Kanakaraj, et al., 2021:492).

AMD consists of maxillary tray with a centrally located adjustable needle (for Gothic arch movement) along with an adjustable lip support flange and a mandibular tray which comprises of a flat occlusal tracing plate. An additional occlusal plane orientation ruler can be attached to the maxillary AMD to help align the maxillary teeth with the pupillary line(Kanakaraj, et al., 2021:492).

Maxillary AMD is coated with adhesive, filled with AvaDent recording material, then seated in the mouth to record the morphology of the maxillary arch and stabilize the tray. The mandibular AMD is then covered with adhesive, filled with Ava Dent registration material, and then the maxillary needle is placed in the mouth parallel to the other tray and in contact with the mandibular movement plate(Kanakaraj, et al., 2021:492).

Occlusal vertical size is determined by evaluating the vertical dimension when existing prostheses are in occlusion or by conventional methods. The adjustable screw is used to move the central bearing pin up or down until it contacts to mandibular movement plate in appropriate vertical dimension. The upper lip support flange in maxillary AMD is then adjusted to obtain an adequate lip support. Gothic arc tracing occurs when the patient makes protrusive and lateral mandibular movements. The needle on the maxillary tray draws the lines on the flat mandubular movement plate. An arrow sign is obtained where the apex of the arrow indicates the centric relation position(CR). Using a round acrylic resin bur on the movement plate, a small indentation is made corresponding to the apex of the gothic arc arrow and the tip diameter of the needle. The mandible is then maneuvered in a position where the needle hits the recess to maintain the centric relationship position. In the next step, the Ava Dent ruler is placed in the maxillary AMD and orient it so that it is parallel to the interpapillary line, and the angle is noted to help guide the producer in the laboratory. The midline, smile line is signed, and tooth size and position of the pink denture base around the cervix of the teeth are selected from the teeth selection shape tabs(Kanakaraj, et al., 2021:492).

As the needle hits the indentation in the movement plate, the mandible is directed to the CR position. An interocclusal recording material is injected into the space between them to secure the maxillary and mandibular AMD trays in place. Definitive impression and attached AMD trays are

disinfected and sent to the producer for additional processing. Prostheses are scanned with a laser and designed virtually. Recesses are milled on the prosthesis base where the prosthetic teeth will be fully seated. Prosthetic teeth are fixated using a suitable bonding mechanism(Kanakaraj, et al., 2021:492).

Second Appointment (Optional): The trial prosthesis is required to validate and evaluate the prosthesis design before milling the final prosthesis. Two types of try in dentures are existing:

- Advanced trial prosthesis: Milled bases with recesses into which the prosthetic teeth are fixed with wax(advanced try-in denture (ATI).
- Biofunctional trial prosthesis milled from full resin: Many tooth shades are available (BTI—Bouma try-in) (Kanakaraj, et al., 2021:492).

At this appointment, the prosthetic teeth can be adjusted in wax by repositioning if necessary. In the trial prosthesis, adjustments can be made by repositioning the teeth in the wax to meet the needs of the patient(17). If necessary, the essential corrections are made in the final prosthesis and sent back to the producer for the final prosthesis manufacturing(Kanakaraj, et al., 2021:492).

Third Appointment: CAD / CAM complete denture placement is almost the same as placement of a conventionally manufactured complete denture. Pressure indicator paste or Fit Checker ™ (GC America, Alsip, IL) is used to assist in making the necessary adjustment to the fit of hollow surfaces to the mucosa. Occlusal adjustment may be necessary and can be done in the mouth. Severe disparities in occlusal contacts between prostheses can be regulated after clinical remount process(Baba, 2016:203–208).

2) The Whole You Nexteeth system (Previously DENTCA™)

Prosthetic trays are used in the production and design of prostheses. CAD / CAM prostheses are made with the help of DENTCA, Inc and DENTCA's sister company Whole You, Inc. The CAD part is completed by DENTCA Inc, and the CAM part is completed owned by Whole You, Inc.(Kanakaraj, et al., 2021:493).

Complete dentures are produced in two ways:

- The trial prosthesis is printed and confirm in the patient's mouth and then conventionally processed using a special 3D-printed flask.
- The denture base is printed with a 3D printer and the denture teeth are attached to the printed base(Kanakaraj, et al., 2021:493).

DENTCA trays consist of two parts with removable back segments. These trays are used for definitive impressions as well as for jaw relationship recording. To record the jaw relationship, the full arch impression is divided into sections, the posterior segments are removed and a gothic arch device is connected to it(Kanakaraj, et al., 2021:493). The system to let the construction of full dentures, single arch prostheses and immediate prostheses(Baba, 2016:203–208).

First Appointment: Functional impressions are made using trays and silicone impression material. A single incision line is made in maxillary and mandibular impressions using a surgical knife to separate the posterior segment from the anterior segments(Kanakaraj, et al., 2021:493).

Here, compared to AMD trays in the AvaDent prosthesis system, the needle connected to the mandibular tray at lingual surface and the movement plate to the maxillary tray. The anterior part of the impression is placed in the mouth and the occlusal vertical dimension is determined and adjustments are made if necessary. The anterior part of the impression is placed in the mouth and the occlusal vertical dimension is detected and regulations are made if necessary. Gothic arch movement occurs when the patient makes protrusive and lateral mandibular movements. The needle on the mandibular tray draws the lines on the maxillary flat tracing plate. An arrow sign is obtained where the apex of the arrow indicates the centric relation position (CR). Using a round acrylic resin bur on the movement plate, a small indentation is made corresponding to the apex of the gothic arc arrow and the tip diameter of the needle(Kanakaraj, et al., 2021:493).

In order to stabilize the interocclusal relationship while recording, the mandible is maneuvered in the position where the needle hits the recess to maintain the centric relationship position. Interocclusal registration record material is syringed in the gap between the maxillary and mandibular trays. There is also a lip scale in the system to measure the length of the maxillary lip (between the incisive papillae and the lower border of the upper lip) (Kanakaraj, et al., 2021:493).

Trays and impressions are disinfected and sent to the laboratory for the production of the final prosthesis. Using special computer software, they are scanned for the production of maxillary and mandibular virtual toothless ridges. Unlike the AvaDent system, which uses subtractive manufacturing, the prostheses are designed virtually and the data is transferred to a 3D laser lithography machine that produces trial prostheses using the rapid prototyping process(Kanakaraj, et al., 2021:493).

Second Appointment (Optional): A prosthesis trial may be requested before manufacturing the final digital prosthesis. Phonetic, aesthetic and functional evaluation of stereolithographically printed trial prostheses is

made. At this appointment, the prosthetic teeth are adapted, if necessary, with selective abrasion(Baba, 2016:203–208).

Third Appointment: CAD / CAM complete denture placement is almost the same as placement of a conventionally manufactured complete denture. Pressure indicator paste or Fit Checker ™ (GC America, Alsip, IL) is used to assist in making the necessary adjustment to the fit of hollow surfaces to the mucosa. Occlusal adjustment may be necessary and can be done in the mouth. Severe disparities in occlusal contacts between prostheses can be regulated after clinical re-mount process.

Disadvantages: The choice to specialize the dentures is limited(Baba, 2016:203–208).

3) Ceramill® Full Denture System(Amann Girrbach)

The Ceramill® full denture system (FDS) (Amann Girrbach AG, Koblach, Austria) is a system designed for laboratory technicians. Unlike previously discussed systems, the digital Ceramill system's workflow starts in the lab. It helps dental alingment, milling of trial wax bases, alteration of prosthetic teeth so that they can be placed in the tooth sockets inside the base with wax without additional abrasion(Kanakaraj, et al., 2021:493).

First Appointment: The definitive impressions of the maxillary and mandibular arches are made and sent to the laboratory to production of definitive casts, records bases to maxillomandibular relationship record and a maxillary base plate for facebow transfer. Registration bases are used to save include VDO (vertical dimension in occlusion), midline, smile line, canine positions and facebow transfer(Kanakaraj, et al., 2021:493).

Second Appointment: Facebow and jaw relationship records are made. The smile line, the positions of the canines, the midline and the position of the anterior teeth for proper lip support and optimum aesthetics are defined(Kanakaraj, et al., 2021:493).

These records are then sent to the lab. The models are then mounted on an articulator and the mounted models along with the occlusal rims are scanned using an optical 3D scanner (Ceramill Map400, Amann Girrbach AG). Models are also scanned individually. This helps to obtain virtual models and also to transfer the position of the models to the design software (Ceramill Mind / D-Flow; Amann Girrbach)(Kanakaraj, et al., 2021:493).

Virtual design, consists of defining the anatomical structure, tooth arrangement, selection of the appropriate artificial tooth alignment. Designed prostheses are sent to the clinician to confirmation. Once

confirmed, the maxillary and mandibular bases are milled from the gingival colored wax block (Ceramill D-Wax, Ceramill Motion 2; AmannGirrbach). Depending on the clinical situation, prosthetic teeth can be altered by milling the basal surfaces of the teeth. The altered denture teeth are then waxed in position on the wax bases. The trial prosthesis is then sent to the clinician(Kanakaraj, et al., 2021:493).

Third Appointment: Trial prostheses are assessed in the patient's mouth in terms of aesthetics, phonetics and function. After the necessary adjustments are made, it is sent to the laboratory for the production of the final prosthesis. Prostheses are produced with traditional technique(Kanakaraj, et al., 2021:493).

Fourth Appointment: CAD / CAM complete denture placement is almost the same as placement of a conventionally manufactured complete denture. Pressure indicator paste or Fit Checker ™ (GC America, Alsip, IL) is used to assist in making the necessary adjustment to the fit of hollow surfaces to the mucosa. Occlusal adjustment may be necessary and can be done in the mouth. Severe disparities in occlusal contacts between prostheses can be regulated after clinical remount process(Baba, 2016:203–208).

4) Baltic denture system (Merz Dental): This prosthetic system is based on the principle of regulation of bite rims including preformed occlusal arches (BD keys) by relining until the dental arches are placed in the anatomically right 3D location. The process is integrated in just two visits, supplying a good rate of patient pleasure. BDKEY set components contain maxillary and mandibular regulatable base registers with teeth, existing in 8 distinct configurations (different palate width and tooth size S, M, L) (Kanakaraj, et al., 2021:494).

First Appointment: The definitive impressions are made by regulating the BDKEY trays in the mouth. Facial midline, interpapillary line, and camper lines are recorded using a facebow connected to maxillary tray. In addition, it helps in transferring functional and aesthetic components from the patient to the design software. BDKEY Lock is a specific apparatus that helps to record the jaw relationship. In general, assessment of aesthetics, lip support, tooth arrangement and interocclusal space are possible due to the fact that the teeth are on the trays. These also work like a prosthesis trial(Kanakaraj, et al., 2021:494).

The records are sent to the laboratory for scanning and virtual designs of prostheses by the BDCreator software. After design confirmation, the prostheses are milled from milling blocks made of polymethyl methacrylate (PMMA) (Kanakaraj, et al., 2021:494).

Second Appointment: Placement process are similar to traditional prostheses. Occlusal regulates are made in the patient's mouth or in the laboratory by remount procedure(Kanakaraj, et al., 2021:494).

5) Wieland denture system (Wieland dental + Technik Ivoclar Vivadent)

This system consists of a five-axis milling machine unified with a lab scanner and design software (3shape ™). The system protocol includes 3 appointments, excluding trial appointments(Kanakaraj, et al., 2021:494). The system to let three methods of obtaining clinical records:

- digitally designed individual impression trays integrated with bite plates
- digitally designed and milled individual wax rims
- reproduction of available prostheses(Baba, 2016:203–208).

First Appointment: Anatomical impressions of the maxillary and mandibular arches are made. A special apparatus called the centric tray (Ivoclar Vivadent) is used for recording the vertical dimension and temporary jaw relationship. A specific arc transfer instrument called UTS CAD is used to define the provisional occlusal plane. The UTS CAD helps in measuring the Camper line and interpupillary line. Preliminary impressions, centric tray and the Camper line and interpupillary measurements are sent to the laboratory technician(Kanakaraj, et al., 2021:494).

The records are scanned and the data transferred to the software to virtually design the prosthesis. Individual impression trays are integrated with occlusal plates designed with a uniform offset (for impression material) and a recess to let stabilization of Gnathometer CAD (Ivoclar Vivadent). Gnathometer CAD helps to record Gothic arc movement and CR(Kanakaraj, et al., 2021:494).

Second Appointment: Functional impressions are made with milled individual impression trays. The occlusal plane is confirmed using UTS CAD. Gnathometer CAD connected to individual trays. The patient's midline, smile line and distance lines from canine to canine are created. VDO and CR are recorded using traditional techniques(Kanakaraj, et al., 2021:494).

The functional impression and records are scanned and data transferred to the software. The occlusal plane is defined, prosthetic teeth are selected and virtual tooth alignment is done. Trial prostheses are produced from the PMMA block(Kanakaraj, et al., 2021:494).

Third Appointment: Trial prostheses are placed in the patient's mouth to evaluate aesthetics, function and phonetics. If necessary, adjustments are made and final prostheses are produced.

Fourth Appointment: CAD / CAM complete denture placement is almost the same as placement of a conventionally manufactured complete denture. Pressure indicator paste or Fit Checker ™ (GC America, Alsip, IL) is used to assist in making the necessary adjustment to the fit of hollow surfaces to the mucosa. Occlusal adjustment may be necessary and can be done in the mouth. Severe disparities in occlusal contacts between prostheses can be regulated after clinical re-mount process.

Disadvantages: Does not to let the production of customized full arch prostheses(Baba, 2016:203–208).

6) Vita Vionic system

This system supplys materials for open CAD / CAM systems. Digital design and manufacturing can be facilitated by non-system native scanners, software, and milling machines. The system is responsive and can be regulated by the protocol proverbial with the user. Thus, the traditional prosthesis manufacturing protocol can be applied with five steps and a shortened session(anatomical impression, functional impression plus determination of vertical and maxillo mandibular jaw relationship, prosthesis placement). The impressions, models or records are saved traditionally and then digitalized. If trial prosthesis is required, it can be milled from wax discs produced by VITA.

Disadvantages: The digitization procedures is not specialized for the entire system(Kanakaraj, et al., 2021:494).

Advantages of CAD / CAM Complete Dentures

1) Decreases the number of patient appointments for elderly patients who have difficulty traveling to and from the dental office.

2) The decrease in clinical working time for the production of complete dentures reduces the overhead of the clinician and increases profitableness.

3) All of the cumulative data, the produced images and regulations can be digitally recorded and used for future production of an extra prosthesis, in the event of loss of the prosthesis or a surgical/radiographic templet.

4) Laboratory working time procedures are decreased or removed, to let the dental technician to make reproducible, effective and correct dentures.

5) The use of prepolymerized acrylic resin by some producers for manufacturing prostheses base supplies excellent conformity and durability

compared to traditionally processed bases. Milled prepolymerized acrylic resin does not show polymerization shrinkage.

6) Independent studies have shown that prepolymerized acrylic resin (PAR) contains less residual monomer and is more hydrophobic than traditionally cured acrylic resin. PAR decreases the potential for infection due to fewer microorganisms (ie C. albicans) adhering to the prosthetic bases (Baba, Goodacre, Kattadiyil., 2015:109)

7) Prepolymerized acrylic resin block material produced under high temperature and pressure is the risk of a allergic reaction is also much lower due to less monomer and minimal porosity.

8) The occlusal fit of the prosthesis can be corrected with minimal adjustments.

9) Total prosthesis is a non-invasive and reversible treatment. In cases where it fails, the prosthesis prepared with CAD / CAM does not meet the needs of the physician and patient. It can be converted to conventional technique by the artificial teeth on the milled prosthesis base are removed(Arslan, 2016:13).

Disadvantages of CAD / CAM Complete Dentures

1) Balanced prostheses are difficult to obtain with dental software used for digital design of complete dentures. Clinically remount can done , if necessary to restore the balance of the denture teeth.

2) Before the clinician feels that he/she has mastered any system, he/she has chosen to manufacture digital prostheses, he/she must make several CAD / CAM full dentures to refrain disillusionment and unwanted consequences.

3) Giving up the trial prosthesis visit can bereave the clinician of the chance to make necessary adjustments for assess aesthetics and phonetics(Baba et al., 2015:110)

CONCLUSİONS

Conventional method for total prostheses requires experienced prosthesis specialists and dental technicians. Also, many patient's appointments and a large amount laboratory work is required. Many times, especially for elderly patients a hospital visit can be uncomfortable. In addition, acrylic resins, does not meet all the requirements for ideal denture base. Integration of CAD / CAM technology into denture design and manufacturing help to improve the quality of prostheses and to facilitate laboratory work. CAD / CAM technology eliminating by shortens time-consuming laboratory procedures thus ensures that repeatable, precise and

smooth prostheses. With the incorporation of CAD / CAM technology into the design and manufacture of full dentures, helps to simplify laboratory work and standardize the production of full dentures. The use of CAD / CAM technology in the production of complete dentures is provides positive benefits for both the patient and the practitioner. Reduction of polymerization shrinkage resulting in increased compliance, development of registered digital data, easy production of replacement dentures and newly placed prosthesis reduction in patient adaptation time is among the advantages it provides. Each CAD / CAM system has its own advantages and disadvantages, common advantages; reduction of session protocols, reduction of residual monomer and reduction in polymerization shrinkage, etc. Different CAD / CAM systems vary according to the number of patient visits and the recording method of occlusal vertical dimension, midline and maxillo-mandibular jaw relationship. These systems also differ in the trial options provided by each. System selection depends on the dentist's prosthetic expertise, prosthetic individualization requirements and yield rate. Early scientific evidence supports the predominance of CAD / CAM complete dentures, although there is more evidence regarding the material, certain features need to be developed.

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Chapter 6

IRON HOMEOSTASIS AND OVERVIEW OF IRON DEFICIENCY ANEMIA

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Iron Homeostasis and Overvıew of Iron Deficiency Anemia

Introduction

The metallic element iron is essential for all organisms' growth and survival. Iron is the fourth most common mineral on the earth's surface and the most abundant metal in the earth's crust. Earth's crust contains approximately 4-5% iron. There are two forms: water-soluble Ferrous iron (Fe2 +) and water-insoluble Ferric form (Fe3 +). Iron is not only participating in the synthesis of hemoglobins and myoglobins that provide oxygen transport and storage, but it is also a critical cofactor in the structure of enzymes that play a critical role in oxidation-reduction reactions cytochrome oxidases, catalase, and peroxidases. However, the high amount of iron can cause cellular and tissue damage by creating free oxygen radicals. Therefore, keeping the iron level at the optimal level in the organism is of vital importance. The total amount of iron in the body for an adult male is approximately 50 mg/kg, and for an adult female is 40 mg/kg. Distribution of body iron; While the vast majority (60-70%) participates in the structure of hemoglobin, which is the oxygen carrier to tissues, 20-30% of it is found in the storage proteins ferritin and haemosiderin in the reticuloendothelial system. A minimal amount of iron in the body is about 3-4 mg in plasma and extracellular fluids bounds to transferrin, the transport protein (Bothwell, Charlton, Cook, & Finch, 1979). The liver is the most crucial location of the body iron stores and plays a vital role in the absorption of iron from enterocytes and circulating iron. Macrophages serve as another critical iron storage location. Iron homeostasis requires strict regulation. Excess iron causes cell death and toxicity through free radical formation and lipid peroxidation due to the pro-oxidant charecter of iron. This chapter will first review iron homeostasis's basic elements: absorption, transport, pathogenesis, clinical features, and iron deficiency treatment.

Normal Iron Homeostasis

Iron is a crucial element because it is essential for erythropoietic function, oxidative metabolism, and cellular immunity. Regulation of absorption of dietary iron from enterocytes plays a critical role in iron homeostasis, as no active excretory mechanism for iron in humans. Excretion of iron through exuviation of dead skin, or gastrointestinal mucosal cells, is negligible. The regulation of iron absorption is mainly determined by the level of iron stores and the need for erythropoiesis. Serum ferritin level is the most reliable indicator of iron storage in healthy individuals, and body iron levels play a more dominant role in the regulation of iron absorption (Cook, Lipschitz, Miles, & Finch, 1974).

Dietary iron amount, gastric pH, chelation of dietary iron, reduction of intestinal cells, intestinal motility, body iron stores, and hypoxia affect iron absorption. Combination use of iron and some medications may affect iron absorption. The absorption of iron is reduced using drugs containing antacids, magnesium, calcium, or zinc. Iron reduces the absorption of drugs such as bisphosphonates, tetracycline, and quinolone.

Molecular Mechanisms of Mucosal Iron Absorption

The regular western diet contains 15-20 mg of iron in total, and about 10% of it is in heme form. Daily 1-2 mg is absorbed mainly from the duodenum. The absorption of iron into the enterocyte is Ferrous Fe2 + and enters through the apical surface. The most critical control point of iron homeostasis is by controlling iron absorption from enterocytes. While ascorbic acid increased erythropoiesis and decreased iron stores, increase iron absorption, anti-acid drug use, aluminum and zinc-rich diets are conditions that reduce iron absorption. Ferric iron (Fe + 3) and ferrous iron (Fe +2) are taken into intestinal cells by separate transport pathways. Ferric iron in the diet is reduced to ferrous iron by duodenal ferric reductase at the brushed border of the duodenum. This transformation both increases the solubility of iron and facilitates its absorption into enterocytes by a divalent metal ion transporter protein (DMT1(Beaumont et al., 2006; Iolascon et al., 2006). DMT1 expression is upregulated in iron deficiency. Ferrous iron in enterocyte cytosol; can be stored in the form of ferritin, discarded during exfoliation of enterocytes, or transferred from the basolateral membrane to the plasma to meet cellular requirements. Iron transport occurs from the basolateral membrane of enterocytes to the portal system with ion transporters as Ferrroportin, IREG1, and MTP1 (Metal Transporter Protein 1). The cells that supply iron to the plasma (enterocyte, hepatocyte, and macrophages) perform this process through ferroportin, which makes ferroportin a vital determinant in iron homeostasis. Ferric form oxidation is performed by hephaestin to transfer iron to plasma by ferroportin, and its ferric form binds to transferrin, the plasma iron carrier.

Hepcidin is a peptide produced in the liver consisting of 25 amino acids. Hepcidin Binds to α2-macroglobulin and albumin in plasma (Itkonen et al., 2012; Peslova et al., 2009). Hepcidin mainly regulates iron homeostasis by binding to ferroportin. It demonstrates this regulation by reducing the absorption of dietary iron from enterocytes and by inhibiting the transfer of storage iron from macrophages and hepatocytes to plasma. The absolute effect of hepcidin is the reduction of plasma iron (Ganz, Olbina, Girelli, Nemeth, & Westerman, 2008; E. H. Kemna, Tjalsma, Podust, & Swinkels, 2007). The clearance of hepcidin is through the

kidneys. Urinary hepcidin level correlates with plasma hepcidin level in the absence of kidney disease (Ganz, Olbina, Girelli, Nemeth, & Westerman, 2008; E. H. Kemna, Tjalsma, Podust, & Swinkels, 2007). Urine hepcidin level, which can be measured by the ELISA method, is correlated with plasma hepcidin level in the absence of kidney disease. Due to the highly dynamic and many different arrangements, the correct interpretation of hepcidin measurements should be made considering all clinical findings. For example, an inflammatory state may coincide with severe iron deficiency, where hepcidin levels are expected to be low hepcidin level. (Peters, Laarakkers, Swinkels, & Wetzels, 2010). Chronic kidney disease, infections / inflammatory disorders, red blood cell transfusions, iron refractory iron deficiency anemia (IRIDA) are among the leading clinical conditions that cause raised hepatic synthesis (E. Kemna, Pickkers, Nemeth, van der Hoeven, & Swinkels, 2005; Origa et al., 2007; Peters et al., 2010). In clinical cases such as iron deficiency anemia, Hepatitis C virus infection, and chronic liver disease, administration of erythropoietic stimulating agents, causes suppressed hepatic hepcidin synthesis (Girelli, Nemeth, & Swinkels, 2016). Cellular regulation of hepcidin expression occurs by amplified and transcriptional inhibition signals. BMP6 is a regulator of hepcidin production in the liver. BMP6 activates the BMP signaling pathway by binding to hepatic BMP hemojuvelin (HJV) and BMP receptors, increasing hepcidin expression and decreasing plasma iron (Ganz, 2013; Lane et al., 2015). Mutations in HJV and hepcidin gene (HAMP) halt hepcidin synthesis, leading to severe iron overload and juvenile forms of hemochromatosis (Zhao, Zhang, & Enns, 2013). Hypoxia-inducible factors (HIFs) increase the transcriptional synthesis of receptors such as iron-related proteins divalent metal transporter 1 (DMT1), ferroportin 1 (FPN1), and transferrin receptor (TfR). Thus, it plays a role in the local regulation of iron. Cytoplasmic iron regulatory proteins (IRP) interact with the iron regulatory elements (IRE) on the m RNA of the key proteins of iron metabolism and provide posttranscriptional control (Gulec, Anderson, & Collins, 2014).

Iron Transport and Intracellular Iron Homeostasis

Transferrin binds to iron in ferric form with a high affinity to protect it from the toxic effect of free iron in plasma. Transferrin binds to ferric iron form with a high affinity. Thus, it is protected from the toxic effects of free iron in plasma. In order to bind iron to transferrin, it must be converted to ferric form and oxidized. The copper-containing ferroxidase enzyme and ceruloplasmin play essential roles in this process of oxidation and binding to transferrin.Transferrin in the plasma binds to the transferrin receptor (TfR), which is synthesized at a level determined according to the intracellular

iron requirement in the cell membrane and located on the cell surface. TfR has two different isoforms as transferrin receptor 1 (TfR1) and transferrin receptor (TfR2), which are encoded by two separate genes. TfR1 is found in all cells.TfR2 is found only in hepatocytes, in the duodenal crypt cells, and in erythroid precursor cells. TfR-2 regulates hepcidin expression in hepatocytes, is essential for liver iron stores signals, and controls iron supply by regulating the use of erythropoiesis in erythroid precursor cells (Girelli, Ugolini, Busti, Marchi, & Castagna, 2018). Molecular control of iron at the cellular level; Synthesis of all significant proteins involved in the transport, storage and utilization of iron is regulated by intracellular iron at the posttranscripsional level. The expression of transferrin receptor and ferritin is maintained by iron regulatory proteins (IRP1 and IRP2) and the iron-responsive element (IRE) signaling pathway.In low iron states, IRP1 and IRP2 bind with high affinity to IRE in ferritin mRNAs and inhibit TfR mRNA translation, causing cellular uptake of iron and preventing storage iron formation. In increased iron, IRP1 and IRP2 decrease affinity for IRE in ferritin mRNAs and induce TfR mRNA translation. Thus, ferroportin synthesis decreases, and iron uptake into the cell decreases, leading to an increase in storage iron formation.

Iron Recycling

The lifetime of erythrocytes is approximately 120 days. A signal generated from the integrated membrane protein band 3 appears on the cell surface of aging erythrocytes, and elimination is by phagocytosis by macrophages in the liver and spleen (Ganz, 2013). The iron in the macrophages leaves into the plasma via ferroportin. With transferrin, the most crucial plasma iron carrier, iron flows into the bone marrow reused in erythropoiesis. Approximately 25 mg of iron per day recovered from the macrophages to the bone marrow, which is considerably greater than 1-2 mg of iron taken with a daily diet and coming into the bone marrow(Ganz & Nemeth, 2012). The most important part of in vivo iron regulation is the reuse of iron in erythrocytes, mainly controlled by hepcidin metabolism.

Diagnostic Methods for Evaluation of Iron Status

While body iron can be measured using a variety of methods, there is no single ideal indicator for evaluating iron status in different clinical situations. The total amount of storage iron is related to the level of ferritin and hemosiderin (Addison et al., 1972; Saito, 2014). The serum ferritin level may not always indicate the actual iron level. Higher values are measured in various inflammatory, liver disease, and malignant conditions (Beaumont et al., 1995). Also, there is no linear relationship between ferritin level and stored iron (Saito, 2014). In reticuloendothelial macrophages and

hepatic parenchymal cells, cellular ferritin is converted to hemosiderin. Another diagnostic method that determines the total amount of storage iron is invasive bone marrow examinations. Serum iron, total iron-binding capacity (TIBC) of transferrin, and serum transferrin receptor (TRs) are evaluated to determine the iron supply to tissues. Transferrin saturation (TS) is obtained by dividing serum iron by total iron-binding capacity, and a TS of less than 15% indicates iron deficiency to support normal erythropoiesis. *Increased serum transferrin receptor concentration* is a sensitive indicator showing decreased tissue iron status. Plasma ferritin concentration reflects the most important indirect information of iron storage in the body. However, ferritin increases with age, liver disease, and inflammation. In these cases, the measurement of total body iron, TS and TRs becomes more valuable (Weiss & Goodnough, 2005). Various novel markers are available with iron supply for erythropoiesis. The hemoglobin content of reticulocytes (CHr), reticulocyte hemoglobin equivalent (Ret-He), and hypochromic circulating RBCs (% HRC) tests are available to determine the iron required for erythropoiesis. HRC values above 6% are significant in early finding of insufficient iron supply. The CHr or Ret-He parameter helps determine the iron status in chronic kidney injury treated with erythropoietin (Tsuchiya et al., 2005).

Iron Overload (Hemochromatosis)

Hemochromatosis is an inherited or acquired disorder of iron metabolism. People with hemochromatosis accumulate more iron than their body needs. Eventually, iron overload can lead to dysfunction and failure of many organs, including the heart, liver, and pancreas (Pietrangelo, 2016). Iron overload in the brain can cause disorders characterized by movement disorders and other neuropsychiatric findings (Singh et al., 2014). Iron and iron-derived reactive oxygen species have been demonstrated in the etiology of acute kidney injury (Paller, Hedlund, Sikora, Faassen, & Waterfield, 1988; Wang et al., 2001). Iron overload causes cardiomyopathy by causing damage to the cardiac electrical pathway in the heart and myocardial fibrosis (Murphy & Oudit, 2010). The most prevalent genetic disorder of iron overload is hereditary hemochromatosis (HH) (Adams, 2015). Acquired iron overload may result from dyserythropoietic syndromes such as thalassemia major and intermedia, or chronic hemolytic anemia, transfusion-dependent anemia, chronic liver disease (Kowdley, 2016).

Hereditary Hemochromatosis

The most common mutation that causes HH is the HFE gene mutation. Other causes of hemochromatosis are mutations in genes

encoding proteins involved in iron metabolisms, such as hemojuvelin (HJV), hepcidin (HAMP), transferrin receptor 2 (TFR2), and ferroportin (SLC40A1). In the classification of genetic iron overload diseases, type 1 defines the classic HH with an HFE gene mutation (Ahmad et al., 2002). Type 2 includes two genetic mutations called juvenile hemochromatosis, characterized by heavier iron accumulation at an early age. Type 2A is the type containing hemojuvelin (HJV) mutation, while Type 2B is the type of hepcidin with a HAMP gene mutation with severe loss of function (Papanikolaou et al., 2004). Type 3 is a disease type with transferrin receptor-2 TfR2 mutation seen in adulthood and similar to the classic HFE gene mutation (Kawabata et al., 2005). Type 4 is a disease of excessive iron overload caused by ferroportin mutation.

The main goal in therapeutic phlebotomy is to keep serum ferritin less than level 100 ng / ml and TS <50%. Afterward, lifetime phlebotomy should be continued every 3-4 months to keep the ferritin level <100 ng/ml (Palmer et al., 2018). While there is no improvement in arthritis and hypogonadism with phlebotomy, there is an improvement in the regulation of diabetes. Iron chelation therapy in HH is an alternative treatment for patients who cannot undergo phlebotomy due to severe heart disease and in patients with acquired hemochromatosis. Liver transplantation should be considered in patients if diagnosis and treatment are delayed and complicated by end-stage liver failure (Feldman, Scharschmidt, & Sleisenger, 1998).

Acquired Iron Overload

Acquired iron overload is the inevitable end of transfusion-dependent chronic diseases. The most prevalent hematological diseases are hemoglobinopathies and sickle cell diseases (Porter & Garbowski, 2014; Williams & Weatherall, 2012). Thalassemia major is one of the diseases in which the effects of acquired hemochromatosis on the heart, liver, and endocrine organs are most clearly determined. The effects of secondary iron accumulation are in adult patients frequently seen due to the increased need for transfusion in myelodysplastic syndromes, myelofibrosis, and leukemias.

Mechanisms of Iron Toxicity

Increased LIP causes redox reactions that produce reactive oxygen species (ROS), resulting in lipid peroxidation, growth factor-beta 1 (TGFβ1) mediated cell death, and fibrogenesis. ROS also damages DNA and accelerates apoptotic death by directly activating caspases (Le Lan et al., 2005)

Iron Deficiency and Iron Deficiency Anemia

Etiology and Pathogenesis

The World Health Organization reported that in 2011, they estimated the worldwide prevalence of anemia at 42% in children, 29% in non-pregnant women, and 38% in pregnant women. The highest prevalence is observed in preschool children (47.4%), the lowest in males (12.7%) (WHO, 2015). Iron deficiency (ID) continues to be the leading cause of anemia, which has significant adverse effects on global health. Blood loss is the primary cause of the iron deficiency. Other important causes of iron deficiency are malnutrition, malabsorption, and increased physiological requirements. The average daily iron loss in healthy men is less than 1.0 mg/day. The average daily iron loss in women with regular menstruation is about 1.5 mg/day. The most common pathological reason is blood loss (Camaschella, 2015). Iron deficiency may be a sign of gastrointestinal malignancies, autoimmune diseases, or infections in postmenopausal women and adult men. Conditions such as pregnancy, adolescent growth, lactation are the causes of iron deficiency due to increased iron need. Infants whose diets occur mainly cow's milk often become iron deficient. Malnutritions is a rare reason for ıron deficiency for adult. Iron-resistant iron deficiency anemia (IRIDA) is a rare autosomal recessive inherited disease of iron deficiency. IRIDA causes an increase in hepcidin due to mutation in the TMPRSS6 gene and blocks intestinal iron absorption (Heeney & Finberg, 2014).

Clinical Manifestations

Iron deficiency anemia can show different clinical features in young people and elderly patients. Fatigue is the most common nonspecific symptom in young people. Cardiovascular symptoms such as dyspnea, angina, or heart failure are prominent in elderly patients (DeLoughery, 2017). Children are susceptible to infections in iron deficiency. Thus, the possibility of meningitis, pneumonia, and gastroenteritis infections increases. The physiopathology of the decreased immune system response is related to disorders in leukocyte functions. In children with iron deficiency, disorders occur in physical and mental development. Many studies have shown that Cognitive deficits can be irreversible with appropriate iron supplementation while physical developmental impairment improves (Deinard, List, Lindgren, Hunt, & Chang, 1986; Lozoff, Jimenez, & Wolf, 1991). However, there are some clinical findings particular to iron deficiency. Pica may be seen as a desire to chew on clay (geophagia) or ice (pagophagia). Rarely, patients may experience

concave nail beds (koilonychia), angular cheilitis, or an esophageal Web (Plummer-Vinson syndrome).

Diagnostic parameters

There is no anemia in the first clinical-stage of latent iron deficiency, hemoglobin hematocrit, and serum iron levels remain normal. However, there is evidence of iron deficiency, such as the absence of iron stores (ferritin and hemosiderin) in the bone marrow and low serum ferritin (Beutler & Waalen, 2006). First, in a persistent iron deficiency, mean cell volume (MCV) decreases, followed by a reduction in mean corpuscular hemoglobin (MCH), hematocrit, and hemoglobin concentrations. The red cell size measured by the red cell volume distribution width (RDW) is significantly variable due to the coexistence of small and normal-sized cells (Tsuchiya et al., 2005). During this time, the total iron-binding capacity (transferrin) increases, causing transferrin saturation to decrease. The peripheral blood smear reveals microcytic, hypochromic, and aberrantly shaped red cells, including “pencil” forms.

Differential Diagnosis

Many conditions cause microcytic hypochromic disorders, including iron deficiency.

Chronic disease anemia, IRIDA, Atransferrinemia, Aceruloplasminemia are other causes of the microcytic hypochromic disorder. Among microcytic hypochromic diseases, while storage iron decreases in iron deficiency anemia, storage iron is either normal or increased in all other disorders (Campion & Deloughery, 2014).

Functional iron deficiency (FID) is the inability to provide sufficient iron to support erythropoiesis due to inadequate mobilization of iron when the iron stores in the body are within normal or high limits. In chronic renal disease, malignancies, and other diseases with chronic inflammation, primarily increased levels of IL-6 and hepcidin, inhibit iron absorption and transport of available iron into the bone marrow, leading to functional iron deficiency. Generally, patients with chronic infection, chronic inflammatory disease, or malignancy typically develop within a few weeks and are mild to moderate in severity. Red cell zinc protoporphyrin concentration is a sensitive parameter for evaluation FID(D. W. Thomas et al., 2013). There are studies showing that patients with functional iron deficiency utility from parenteral iron therapy (L. Thomas et al., 2005).

Management and Treatment

ID/IDA is due to many various reasons and is not the definitive diagnosis. To effectively management of ID/IDA requires identification and treatment of the underlying etiology. In most patients, ID/IDA is restored with oral iron therapy due to a cost-effective and straightforward approach. Iron salts are commonly used in oral iron therapy, and the most common of these is ferrous sulfate. Ferrous gluconate is another oral form. Ferrous gluconate is preferred in children and in cases where fewer side effects are desired. The use of iron-polymaltose complex, which has the least food-drug interactions, and ferrous fumarate, which allows slow iron release, may reduce the side effects of oral iron therapy. Oral therapy is not always well-tolerated. Nausea, vomiting, diarrhea, constipation, and metallic taste are the most common side effects. In such cases, the effectiveness of the treatment decreases. Other situations in which oral therapy is less effective may indicate previous gastrointestinal surgery, inflammatory bowel disease, mild to moderate anemia, congestive heart failure (Qaseem, Wilt, & McLean, 2013). Many parenteral iron complexes are currently available that can be administered intramuscularly or intravenously. Except for some cases especially the risk of allergic-anaphylactic reaction and the cause of cost are not considered as first-line therapy. Parenteral iron therapy is used as a convenient therapy in inflammatory bowel disease (Avni, Bieber, Steinmetz, Leibovici, & Gafter-Gvili, 2013; Stein, Plantz, Maxwell, Mamula, & Baldassano, 2018), chronic kidney disease (Ratcliffe et al., 2016), chemotherapy-induced anemia (Gafter-Gvili et al., 2013), and those who have undergone previous gastrointestinal surgery (Auerbach, Muñoz, & Macdougall, 2018). Ferric sucrose and ferric gluconate require repeated infusions. The use of high molecular weight iron dextran has serious adverse reactions, including dyspnea, wheezing, chest pain, nausea, anaphylaxis, so a low molecular weight iron dextran preparation is preferred. Iron isomaltose is administered by intravenous (IV) infusion or slow IV push. An ıron–sucrose complex and iron(III)-hydroxide carbohydrate complex are given by intravenous infusion. The iron-sucrose complex should be used in a smaller dose in those who receive erythropoietin therapy due to chronic kidney disease(Girelli et al., 2018). Avoiding iatrogenic iron loading is an important issue that the physician should keep in mind in intravenous iron treatment. Parenteral iron effectively improves physical performance and the functional status in chronic heart failure, independent of correcting anemia. Even if the ferritin value was high in these patients, it was more effective than placebo in patients receiving parenteral iron therapy (Anker et al., 2009; Ponikowski et al., 2015). Another important issue is that anemic perioperative patients should have higher mobility and

morbidity. Preoperative parenteral iron therapy reduces the frequency of intraoperative anemia and allogeneic blood transfusions. In ID-IDA treatment, it is recommended to avoid transfusion of blood cells without hemodynamic instability (Callum, Waters, Shaz, Sloan, & Murphy, 2014).

Summary Points

The metallic element iron is essential for all organisms' growth and survival. Iron is not only participating in the structure of hemoglobins and myoglobins that provide oxygen transport and storage, but it is also a critical cofactor in the structure of enzymes that play a critical role in oxidation-reduction reactions cytochrome oxidases, catalase, and peroxidases. However, the high amount of iron can cause cellular and tissue damage by creating free oxygen radicals. Regulation of absorption of dietary iron from enterocytes plays a critical role in iron homeostasis, as no active excretory mechanism for iron in humans. Approximately 25 mg of iron per day recovered from the macrophages to the bone marrow, which is considerably greater than 1-2 mg of iron taken with a daily diet and coming into the bone marrow. While ascorbic acid increased erythropoiesis and decreased iron stores, increase iron absorption, anti-acid drug use, aluminum and zinc-rich diets are conditions that reduce iron absorption. Hepcidin mainly regulates iron homeostasis by binding to ferroportin. It demonstrates this regulation by reducing the absorption of dietary iron from enterocytes and by inhibiting the transfer of storage iron from macrophages and hepatocytes to plasma. Cellular regulation of hepcidin expression occurs by amplified and transcriptional inhibition signals. Iron is absorbed in the duodenum and transferred to transferrin, a plasma transport protein. Fe-transferrin allows free iron to be internalized and released, which can be stored by interacting with certain receptors on cells, used in the production of iron-containing proteins, or exported from the cell via ferroportin. Ferritin and hemosiderin levels represent the total amount of iron in storage. Determination of serum iron, total iron-binding capacity (TIBC) of transferrin, and serum transferrin receptor (TRs) concentration indicate the iron supply of tissues.

The most common mutation that causes Hereditary Hemochromatosis is the HFE gene mutation. Other causes of hemochromatosis are mutations in genes encoding proteins involved in iron metabolisms, such as hemojuvelin (HJV), hepcidin (HAMP), transferrin receptor 2 (TFR2), and ferroportin (SLC40A1). Acquired hemosiderosis is seen in anemic patients who have had multiple blood transfusions. ID/IDA is due to many various reasons and is not the definitive diagnosis. To effectively management of ID/IDA requires identification and treatment of the underlying etiology. Children with iron deficiency may be susceptible to

infections and mental and physical growth retardation. Iron overload can cause liver-heart damage, increased frequency of infections, arthropathy, and many neurological and endocrine disorders. In most patients, ID/IDA is restored with oral iron therapy due to a cost-effective and straightforward approach.

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Chapter 7

EMERGENCY ROOMS IN TURKEY

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The emergency is defined in the dictionary as "a serious, dangerous, unexpected, and often situation requiring immediate action" ("Emergency | Definition of Emergency by Oxford Dictionary," n.d.). In the Turkish Social Security legislation, the emergency is defined as follows: In cases of sudden illness, accident, injury, and similar situations that are required medical intervention within the first 24 hours following the occurrence of the event. These situations are considered to be at risk of losing life and/or health integrity in case of urgent medical intervention or transfer to another health institution. It is the diagnosis and treatment for pandemic cases during the pandemic. For these reasons, the health services provided are considered emergency health services ([Social Security Institution Health Practice Statement] Sosyal Güvenlik Kurumu Sağlik Uygulama Tebliği, 2013). In addition, The Ministry of Health describes the emergency services as follows: The evaluation of the patient by the healthcare personnel in the emergency department in order to protect the patient from complications, morbidity, disability, or death in unexpected situations such as an acute attack of chronic disease, sudden disease, accident, injury. These are the emergency health services provided in inpatient healthcare facilities until the diagnosis is made, medical intervention; moreover, the treatment that will eliminate the life-threatening situations ([Communiqué on Application Procedures and Principles of Emergency Services in Inpatient Health Facilities] Yataklı sağlık tesislerinde acil servis hizmetlerinin uygulama usul ve Esasları hakkında tebliğ, 2009).

Emergency healthcare services in Turkey are primarily provided in emergency services established within hospitals. Although emergency ambulance services are also a part of the emergency health system, most of the diagnosis and treatment processes of patients take place in emergency services. Emergency ambulance services are organized to pick up the patient from the scene in case of an emergency, safely transfer the patient to the hospital's emergency department, and perform the first emergency intervention required during this transfer. In the health legislation, the services provided by the ambulance teams are defined as emergency assistance, and the services provided by the emergency services as emergency treatment ([Emergency Health Services Regulation] Acil Sağlik Hizmetleri Yönetmeliği, 2000). Although primary health care institutions have the essential medical equipment to provide the first response and refer the case in an urgency, emergency treatments are essentially implemented in hospitals.

When looking at the health service infrastructure in Turkey; As of 2019, there are 895 hospitals belong to the Ministry of Health, 68 university hospitals, and 575 private hospitals. In addition, there are 26476 examination units in 7997 family health centers. 524,801,756 (64.6%) of

the 812,903,622 patient examinations conducted in 2019 were performed in secondary and tertiary healthcare facilities (BORA BAŞARA et al., 2021). The rate of physician application per person, which was 3.1 in 2002, exceeded three times this rate as of 2019 and accelerated to 9.8 per year (BORA BAŞARA et al., 2021).

It was improbable that the annual total number of applications to doctor is almost ten times the Turkey population would not be reflected in the emergency services. According to the Ministry of Health data, in the first ten months of 2017, the number of emergency examinations number performed only in adult emergency services of public hospitals was 76,834,439, and this figure constitutes 26% of all examinations performed in the state hospitals (*Top 100 Hospitals in Each Branch-2017 Number of Public Hospitals Examination, Hospitalization, Intensive Care, Surgery, Emergency Service and Births*, 2017). No official data has yet been announced regarding the number of emergency examinations in 2019. Considering that a total of 387.622.848 examinations were performed in public hospitals in 2019, it can be estimated that the number of adult emergency examinations was around 100.000.000. Regarding that Turkey's population was around 83.000.000 in 2019, it can be conceded that ER solely had to examine more patients than the entire country's population (TUİK, 2020).

Regardless of the patient's insurance status or citizenship, all admitted patients can be examined in emergency services in Turkey. Especially if the patients have a disease requiring urgent intervention, the necessary first intervention is performed notwithstanding the patients' financial status. The Ministry of Health and the Social Security Institution provide emergency physicians all kinds of tests, such as blood tests, x-ray, MRIs, CTs, angiography, to diagnose and treat an emergency.

It is essential not to keep patients admitted to the emergency room waiting, and all tests are analyzed as soon as practicable. No appointment mechanism is operated for emergency cases. Whenever everyone assumes that they have an emergency, they can apply to the ER. Also, there is no legal regulation that restricts the number and time of administrations to the ER. Of course, such an unlimited emergency health service delivery opens the door to patient behaviors that will consider abusing. Patients can be encountered who constantly demand health services from the ER without applying to a polyclinic and are admitted to the emergency department dozens of times a year (Durmus & Guneysu, 2021). In this way, the term "frequent users" has become used for patients who apply to emergency services four times or more in a year (Birmingham, Cochran, Frey, Stiffler, & Wilber, 2017; Grover & Close, 2009; LaCalle & Rabin, 2010).

Levels of Emergency Services

Emergency services in Turkey are divided into three levels according to the size of the service they provide, the facilities they have, and their physical size:

First-level emergency services: These are the units where general practitioners provide healthcare services. In cases when specialist experience is demanded, the relevant branch specialist can be reached by phone. In these emergency services, specialist physicians do not keep on-boarding shifts, and they arrive hospital from home if required. When challenged with complex or very urgent cases, they are usually referred to higher centers. These emergency services are found in hospitals with less than 100 beds. Furthermore, they can be up to 400 square meters in size ([Communiqué Amending the Communiqué on the Application Procedures and Principles of Emergency Services in Inpatient Health Facilities] Yataklı Sağlık Tesislerinde Acil Servis Hizmetlerinin Uygulama Usul ve Esasları Hakkında Tebliğde Değişiklik Yapılmasına Dair Tebliğ, 2018).

Second-level emergency services: In these units, emergency healthcare services are presented by general practitioners, and one specialist physician from each of the internal and surgical branches is onboarding duty at the hospital. These services are commonly located in hospitals with 100-300 beds and are 400-800 square meters in size. Computed tomography and ultrasound utilities are available in these emergency services ([Communiqué Amending the Communiqué on the Application Procedures and Principles of Emergency Services in Inpatient Health Facilities] Yataklı Sağlık Tesislerinde Acil Servis Hizmetlerinin Uygulama Usul ve Esasları Hakkında Tebliğde Değişiklik Yapılmasına Dair Tebliğ, 2018).

Third-level emergency services: These units are high-level emergency services that can admit all kinds of emergency cases. They are established in hospitals with more than 300 beds; moreover, they have a minimum area of 800 square meters. In these emergency services, general practitioners and emergency medicine specialists work collectively, and a specialist physician from each branch is onboarding shifts to respond to emergency services demands. These emergency services have personnel support and medical devices that can observe patients in the requirement of intensive care ([Communiqué on Application Procedures and Principles of Emergency Services in Inpatient Health Facilities] Yataklı sağlık tesislerinde acil servis hizmetlerinin uygulama usul ve Esasları hakkında tebliğ, 2009).

Triage System in Emergency Service

Several unrelated cases are applied to emergency services for various reasons, such as easily accessible, serving 24/7, not demanding an appointment, and insufficient primary health care services (Al-Otmy, Abduljabbar, Al-Raddadi, & Farahat, 2020). It is unlikely that non-urgent patients do not apply to these hospital units, which examine millions of patients annually. In research conducted in Turkey in 2006, it was reported that 32.2% of the patients who applied to emergency service were not suitable for emergency service (Ersel et al., 2006). It has been published that patients with different education levels present to emergency services for purposes such as polyclinic density and false perception of emergency (Payza, Karakaya, & Topal, 2020). Emergency services will inevitably be intense due to these inappropriate emergency room applications. As a result of the intensity of emergency services, long waiting times, inadequate medical treatments, increased mortality, and increased health expenses can occur (Salway, Valenzuela, Shoenberger, Mallon, & Viccellio, 2017).

The intensity that occurred in the emergency services made it necessary to classify subjects with and without an emergency health condition at the emergency room entrance. With this purpose called triage, patients are directed to the relevant areas of the emergency department according to the severity of their symptoms and vital signs. In history, triage was used to group French soldiers injured on the battlefield according to the cruelty of their condition, and it has expanded and reached the present day (Yancey & O'Rourke, 2021). It has been announced that even rapid triage performed with a nurse at the entrance of the emergency room led to fewer unsuitable patients in the emergency room observation rooms (Moura & Nogueira, 2020). The triage is to decide which one should be examined first if serious, less severe, and people with no severe symptoms present simultaneously.

The three-color triage is the most generally used emergency room triage method in Turkey. In this system, patients are categorized in three colors as green, yellow, and red. While patients with life-threatening conditions that need to be intervened immediately are in the red color category; patients who have diseases that may be symptoms of a critical illness and may require urgent intervention are in the yellow category. Patients applied to the emergency service outpatient and did not represent any urgent symptoms as the green category. With a change in the emergency legislation in 2018, cases with the color code green were also separated into two subgroups: The patients in the Green 1 category are outpatients with mild symptoms, who need to be examined in the emergency department but are not expected to be life-threatening for up to one hour. The Green 2 category includes cases with no medical disadvantage in

waiting for up to four hours, who have applied to the emergency service with simple symptoms, and can be examined in outpatient clinics to be established outside the emergency service ([Communiqué Amending the Communiqué on the Application Procedures and Principles of Emergency Services in Inpatient Health Facilities] Yataklı Sağlık Tesislerinde Acil Servis Hizmetlerinin Uygulama Usul ve Esasları Hakkında Tebliğde Değişiklik Yapılmasına Dair Tebliğ, 2018). Although it is stipulated by the legislation that other branch specialists examine the patients in the Green 2 category, this system has not been fully implemented in the emergency services.

The Overcrowding of Emergency Department

The congesting of emergency services has commenced becoming a problem in Turkey and other countries of the world. The number of emergency service applications in the USA, which was around 44 million in 1968, approached 134 million in 2013 (Adams, 2013). Around 130 million cases applied to emergency service in 2018 in the USA; furthermore, only 12.4% were hospitalized ("Emergency Department Visits," 2021). It is recognized that patients without hospitalization indication constitute the majority of emergency service admissions. In the UK, emergency room admittances expand with each passing year; in 2019, it was announced that there was a 21% increase compared to the last ten years ("Hospital Accident & Emergency Activity 2019-20," 2020, pp. 2019–2020).

A crowded emergency room can generate some obstacles: The prolonged waiting period of actual patients, the increase of the length of stay in the hospital, leaving of some severe patients from the emergency room because of idling, the decrease in the quality of medical care, the extended waiting time of the ambulances, and the rise in mortality are some of these (Salway et al., 2017).

An equalization needs to be established to assume the population in emergency services. In this equation, patients arriving and departing to the emergency room must be in balance.

Patients applying the emergency room:

- Outpatients,
- Patients arriving by ambulance,
- Patients referred by other physicians.

Patients leaving the emergency room:

- Discharged,
- Those who are hospitalized,

- Those referred to another health institution,
- The deceased.

Two main factors disrupt the balance in this equation: The first is the incapability to restrict outpatients applying, and the second is that hospitals have a limited number of beds for hospitalized patients. It can be estimated that outpatient cases have no urgency to create intensity during emergency service application and examination. On the other hand, the insufficient hospital beds may cause the patients who need to be hospitalized to stay in the emergency room and create density.

CONCLUSION

Emergency rooms have an essential part of healthcare service in Turkey and the world. With the developing world and time passing, applying people to emergency rooms is increasing. Health managers should plan the prevention of overcrowded emergency rooms and uninterrupted provision of health services according to current needs.

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Chapter 8

TRANSCRIPTIONAL REGULATION OF EPITHELIAL-TO-MESENCHYMAL TRANSITION BY TGFβ ISOFORMS

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Introduction

1.1 EMT

An epithelial-mesenchymal transition (EMT) is a process that causes an epithelial cell, which is embedded on the basement membrane via ECM components, to undergo a transition by gaining a mesenchymal phenotype. EMT and MET (mesenchymal-epithelial transition) processes play an important role in organogenesis, embryogenesis, and metastasis of tumor cells. Invasive cancer cells extravasate from the primary tumor site by gaining mesenchymal phenotype and form a secondary tumor in a different site. (Kalluri & Weinberg, 2009) During the transition between epithelial and mesenchymal states, cells preserve their plasticity as the processes are reversible. Throughout the transition, cells gain a hybrid stage in which they co-express epithelial and mesenchymal markers. This mixed epithelial and mesenchymal stage of the cells is present in development, wound healing, especially in cancer metastasis (Liao & Yang, 2017).

1.2 TGFβ

Transforming Growth Factor Beta (TGFβ) is the most recognized and studied driver of the EMT (Moustakas & Heldin, 2016). During EMT, cells lose their attachments and interactions with each other, and alterations in extracellular matrix proteins gain migratory features to become a fibroblast-like mesenchymal phenotype. This switch is caused by transcriptional changes in genes like E-cadherin and N-cadherin (cell adhesion molecules). TGFβ signaling pathway activates the transcription of those marker genes via transcription factors (TFs) like Zeb1,2, Twist1,2, Snail1,2. These EMT-inducing transcription factors, induced by TGFβ, facilitate the acquisition of mesenchymal phenotype and suppress E-cadherin expression, and increasing N-cadherin levels (Loh et al., 2019).

1.3 TGFβ and EMT in Cancer

TGFβ induced EMT is a key indicator of metastasis and invasiveness in many cancer types. (Massagué, 2008). TGFβ acts as a tumor suppressor in early-stage tumors with epithelial cells. TGFβ leads to cell cycle arrest by suppressing cell growth and proliferation in the G1 phase (Iordanskaia & Nawshad, 2011). It causes cell death of tumor cells at an early stage by expressing TGFβ-SMAD dependent apoptotic factors. (Spender et al., 2009). In later stages, tumor cells become resistant to the growth inhibition caused by TGFβ. Thus, cancer cells start to produce TGFβ to enhance their invasive and migratory profile. TGFβ, its downstream signaling

molecules, and receptors are used in clinical trials as a drug target by itself or combined with other cancer therapy methods. Targeting is done by inhibiting receptor binding, blocking the expression of the ligand, and interfering with the translation (Hao et al., 2019). High expression levels of Vimentin (Vim) have been used as an EMT marker as it is associated with cell motility, adhesion, and invasion (C. Y. Liu et al., 2015). It is also used as a bad prognosis factor by causing EMT mediated metastasis in cancers. (Wu et al., 2018). Just as in the case of Vimentin, the results of this study will arise new possible EMT markers and prognosis markers for EMT-related metastasis in epithelial cancers, which will lead to new therapeutic aspects and illuminate the inadequacies in the literature.

Materials and Methods

2.1 Microarray Data Selection

Public microarray data were collected from the GEO (Gene Expression Omnibus) database in the NCBI portal. Chosen data were collected depending on (1) only from the Affymetrix platform, (2) various cancer and normal cell lines from only human and mouse organisms. (3) valid expression levels of epithelial and mesenchymal marker genes like CDH1, VIM, ZEB1, SNAI2 for TGFβ induced EMT presence. (4) at least three repeats of each treatment. (5) clear knowledge of the TGFβ isoform (1-2-3) used.

2.2 Population and Samples of The Study: TGFβ Induced Datasets

In GEO: GDS3710 dataset, Human A549 (Adenocarcinoma) cell line was induced with five ng/mL of TGFβ1 for 72 hours. GPL570, GPL81, and GPL1261 Affymetrix platforms were used for microarray data (Keshamouni et al., 2006). In GEO: GDS4106 dataset, Human Panc-1 (Pancreas) cell line was treated with five ng/mL of TGFβ1 for 48 hours (Maupin et al., 2010). In GEO: GSE13986 dataset, murine mammary gland cells (NMuMG) were treated with five ng/mL of TGFβ1 and TGFβ3 for 24 hours (Z. Liu et al., 2008). In GEO: GSE114761 dataset, human lung cancer cell lines A549, H1944, H292, and H358 were treated with five ng/mL of TGFβ1 for 48 hours (Gordian et al., 2019). In GEO: GSE35830 dataset, human ectocervical epithelial cells were treated with 15 ng/mL of TGFβ3 for 10 hours (Sharkey et al., 2012). In GEO: GSE49644 dataset, human non- small cell lung cancer cell lines (NSCLC) as; A549, HCC827, and H358 were treated with two ng/mL of TGFβ1 for three weeks (Sun et al., 2014). In GEO: GSE40266 dataset, human normal ovarian fibroblast (NOF) line NOF151 was treated with five ng/mL of TGFβ1 and TGFβ2

for 48 hours (Yeung et al., 2013). In GEO: GSE40466 dataset, murine mammary gland cells (NMuMG) were treated with five ng/mL of TGFβ2 for 24 hours (Hussey et al., 2012).

2.3 Differential Expression Analysis

Differential expression analysis was performed using R programming. (Version 3.6.0 for Windows (32/64 bit)) (Amezquita et al., 2020). Data were normalized by the RMA Method using the Affy package of Bioconductor (Gautier et al., 2004). Quality control charts of the Affymetrix microarray datasets were created by using the Simpleaffy package (Miller, 2019). Using pOverA(0.25, log2(16)), filterfun and genefilter functions of Genefilter package, data were filtered (Gentleman et al., 2019). NA values, repeated genes were removed during filtration. Non-significant values based on their P-values were excluded during the analysis. With the Limma package, differential expression analysis was performed (Ritchie et al., 2015). Genes were ranked by their logarithmic fold change values (log2±0.6 and log2±1).

2.4 GSEA and Go Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed with GSEA software and The Molecular Signature Database (MSigDB) gene sets. Data were prepared in GenePattern modules (Reich et al., 2006). 50 Hallmark (H) gene sets from MSigDB collections were used as gene families. Regulated genes were ranked based on their enrichment scores (ES) on the assigned phenotype. Gene sets were filtered with parameters like minimum (50) and maximum gene set sizes (150). False discovery rate (FDR) was determined as less than 25% and P-value as less than 0.01. (Subramanian et al., 2005).

GO (Gene Ontology) enrichment analysis was performed via G: profiler (Raudvere et al., 2019) online tool. Overrepresented genes in the query are analyzed by hypergeometric tests. Significant information based on the logarithmic P values, biological processes, molecular functions, and cellular components are determined. Upregulated genes belonging to each isoform phenotype (TGFβ1, TGFβ2, TGFβ3) from differential expression analysis were used as input queries.

2.5 Cancer Genomics Data Analysis

Cbio portal (Gao et al., 2013) was used for cancer genomic data analysis. Alteration types as mutation, deletion, amplification, expression rates of the TGFβ genes in the cancer types were determined using the portal.

Results and Discussion

3.1 TGFβ has differentially expressed genes that are isoform-specific

Differential expression analysis was performed in R and Bioconductor package of R. Public datasets from the GEO database of TGFβ1, TGFβ2, TGFβ3 induced human, and mouse cell lines were analyzed, normalized, and filtered. Differentially expressed genes (DEGs) genes were ranked based on their fold change values (Table 1). Within the differential expression analysis, regulated genes were intersected to investigate the common genes with β1, β2, and β3 isotypes and genes regulated only in one isoform. For Mus musculus (Mouse) cell line NMuMG, Datasets GSE13986 for β1 and β3; GSE40466 for β2 were used. For Homo sapiens (human) data, datasets GSE35830 (ECT1) for B3, GSE40266 (NOF151) for β1 and β2, and GSE114761 (A549, H358),

GSE49644 (A549, H358) for β1 were used. Upregulated (log2 +0.6) and downregulated (log2 -0.6) genes were separately intersected for mouse and human datasets. In human data, common genes in three isoforms are higher in upregulated values. One hundred seventy-eight upregulated genes in mouse, and 126 upregulated genes in human were shared among all isotypes. One hundred twenty-four genes were specific for TGFβ1 like ID2, CCPG1, NGF, and LOXL1. Three hundred three genes were specific for TGFβ2, such as IDH2, DAP, and TNNT2. Some of the 512 TGFβ3 specific genes were; SOX4, IL6, LAMA3, CDK6, and OVOL1 (Figure 1).

3.2 All three TGFβ isoforms share common differentially expressed genes with human and mouse cell lines.

In the lists obtained from differential expression analysis (log2 fold change ± 0.6), out of 1590 genes from TGFβ1 treated mouse dataset and 2602 genes from human datasets, an intersection of 3 isoforms were checked. 13 genes were common for all isotypes obtained from common genes of mouse and human. Those 13 genes were IL11, PTGS2, PDLIM7, GADD45B, LAMB3, IFIT3, SKIL, ID3, CXCL1, PCDH7, DHRS3, FN1, and MAP3K5. Out of 1590 genes from TGFβ1 treated mouse dataset and 2602 genes from human datasets had 395 common genes. In TGFB2 treated datasets, out of 1151 genes from the mouse dataset and 1093 human datasets, there were 146 shared genes. In TGFβ3 treated datasets, out of 1279 genes from the mouse dataset and 420 genes from the human dataset, 70 genes were common (Table 2).

3.3 There are common, regulated genes among isotypes revealed by gene set enrichment analysis.

Gene set enrichment analysis (GSEA) was performed to reveal the missing points from the single gene expression analysis, such as affected pathways and cellular processes. Overrepresented genes were ranked based on their expression differences and correlations with two different phenotypes (control vs. TGFβ treated). Twenty genes that are on the top of the ranked list based on their enrichment score (ES) in control and TGFβ treated phenotype were listed. Genes like SERPINE1, IL11, TSPAN2, MAF, and CTGF appeared in more than three datasets in TGFβ treated phenotype as upregulated and in all isotypes regardless of the organism (Figure 2).

3.4 Isotype differences in molecular functions, biological processes, and cellular components.

GO (Gene Ontology) analysis was performed via G: profiler. Genes that are upregulated only in certain isoforms were used as queries and analyzed. Each gene set belonging to a certain isotype (TGFβ1, TGFβ2, and TGFβ3) was revealed based on their molecular functions, biological processes that they take part in, and cellular components they belong to. Lists were ranked based on their logarithmic P-values. For the TGFβ1 phenotype, the most significant molecular function is adenyl nucleotide. TGFβ1 is mostly found in the cytoplasm, then membrane-bound organelle as revealed by the cellular component analysis. Biological processes highly correlated with TGFβ1 genes are mitotic cell cycle, cell division. TGFβ2 had molecular functions with high logarithmic significance as enzyme binding, protein binding. For cellular components, TGFβ2 is highly present in intracellular parts. In biological processes, TGFβ2 functions in metabolic processes. Finally, TGFβ3 has similar molecular functions with TGFβ1, like protein binding and ATP binding. TGFβ3 took part in distinct biological processes like cellular response to chemical stimulus. TGFβ3 resides in the cell's intracellular parts.

3.5 TGFβ gene mutations have high alterations frequencies in different cancer types

Cancer genomic analysis was performed via CbioPortal. 74004 samples from 188 studies in TCGA PanCancer Atlas were used in the analysis. Out of 46760 samples, almost 30000 samples were profiled. Alteration frequencies and cancer types are shown. 1503 (3%) of samples were altered in the TGFβ1 gene, and 1079 (2%) were altered in TGFβ2. Bladder/Urinary tract cancer has the highest alteration frequency in the gene; TGFβ1 is amplified 9.7% of this cancer's cases. TGFβ1 is altered in 4.31% of sarcoma cases. 18.9% of the breast cancer cases have TGFβ2 amplification. Endometrial Carcinoma has the highest alteration in the

TGFβ3 gene, 4.27% of the cases have alterations in TGFβ3 (Figure 3). There were 96 missense, 16 truncating, 2 inframe, and a total of 120 mutations on the TGFβ1 gene. Eight mutations are caused by a missense G29R/E amino acid change in non-small cell lung cancer, invasive breast carcinoma, and hepatocellular carcinoma. In the TGFβ2 gene, there were 138 missense, 35 truncating, and 176 total mutations. The highest number of mutations was an R131*/Q amino acid change truncating mutation, which affects colon and colorectal adenocarcinoma. 86 missense, 19 truncating, 4 inframe, and 109 total mutations on the TGFβ3 gene were spotted. In three of the inframe mutations, there was a K38del/N amino acid change on the gene (Figure 4). Some of the cases have alterations in both genes or none of the genes. Thirty-five cases have alterations in both TGFβ1 and TGFβ2. TGFβ1 and TGFβ3 are altered together in 19 of the cases. TGFβ1 and TGFβ2 have more co-occurrence in alteration (Table 3).

Table 1: Top 10 upregulated genes from differential expression analysis. Genes were ranked based on their logarithmic fold change (+log2).

Data Set	Cell LIne	TGFB	Top 10 Upregulated Genes
GDS3710	A549	B1	MAF, IGFBP5,IGFL1,SPOCK1,IL11,DHRS2,INHBA, LINC01279,CDH11
GDS4106	Panc-1	B1	CDH19,COL1A1,FOXS1,LTBP2,THBS1,TAGLIN,OXTR,MYO10
GSE13986	NMuMG	B1	TTR,UGT2B34,SERPINA1E, ALDH1A1, GSTA4,ITIH2, CYP3A13,CYPCL5,VNN,FGB
GSE13986	NMuMG	B3	RNASE4,UGT2B34,SERPINA1C, APOC2, ALDH1A1, SERPINA1E, HPX,TFF2,GSTA2,VNN1
GSE49644	H358	B1	IGFBP5,COL14A1,HS3STSB1,ALPK2,F2RL2,APCDD1L-DT,L1CAM,SPARC, ARHGEF40
GSE49644	A549	B1	INHBA,DNER,CDH11,IL33,VIP,BMP2,IL1A,BHLHE41, ANO4,LCE3D
GSE114761	A549	B1	INHBA,LCE3D,COL15A1,MAF,TNFAIP6,IGHL1,KANK4, COL1A1,SLN,MMP1
GSE114761	H1944	B1	NEDD9,RBP1,MAF,COL1A1,TSPAN2,LUM,PPBP,PTPRR, PMEPA1,LBH
GSE114761	H292	B1	HMGA2,CXCL8,SCEL,PHLDA1,TMCC3,PTPN22,KRT6A, ADGRF1,DNER,FST,SOX2
GSE114761	H358	B1	SERPINE1,MAF,ARHGEF40,MSC-AS1,ACKR3,AL11,LTBP2, LBH,CST6,PDLIM4
GSE35830	Ect1	B3	FN1,SERPINE1,LINC02551,NEDD9,PMEPA1,KANK4,IGFBP3, AKAP12,JUN,IGFBP3
GSE40266	Nof151	B1	COMP,ID4,TSPAN2,NEDD9,PLN,PMEPA1,ITGA4,DHRS2, MYOZ1,LDB3
GSE40266	Nof151	B2	COMP,TSPAN2,ID4,ATP10A,PLN,DHRS2,ITGA4,PMEPA1, RASGRP1,MYOZ1
GSE40466	E1kd	B2	BC080696, TCSTV1,GM20767,PDGFRB,GM12800,AF067061, CEP55, SOX11,EGR2
GSE40466	NMuMG	B2	KLHL30, AK1, LCE1F, CTLA2A, FN1, GJA1, CTSW, ACKR3, NCAM1,CLDN4

Table 2: Number of differentially expressed genes (log2 ± 0.6) common between TGFβ treated mouse and human datasets.

TGFB Isotype	Number of Common Genes
B1	395
B2	146
B3	70

Table 3: Number of cases that are different or co-occurred in each phenotype.

A	B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	p-Value
TGFB1	TGFB2	23754	232	488	35	2.876	<0.001
TGFB2	TGFB3	23805	497	181	26	2.782	<0.001
TGFB1	TGFB3	24054	248	188	19	>3	<0.001

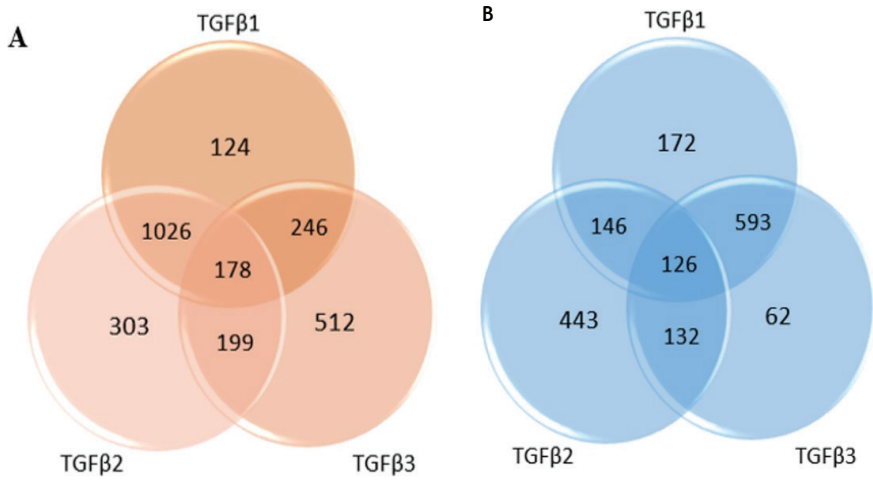


Figure 1: Upregulated (log2 fold change +0.6) genes shared in (A) Mus musculus (NMuMG) TGFβ treated datasets with GEO Accession numbers; GSE13986 for β1, β3 and GSE40466 for β2 were used. (B) Homo sapiens (Human) datasets with GEO Accession numbers; GSE35830 (ECT1) for β3, GSE40266 (NOF151) for β1 and β2 and GSE114761 (A549, H358), GSE49644 (A549, H358) treated with TGFβ1, TGFβ2, TGFβ3 from differential expression analysis.

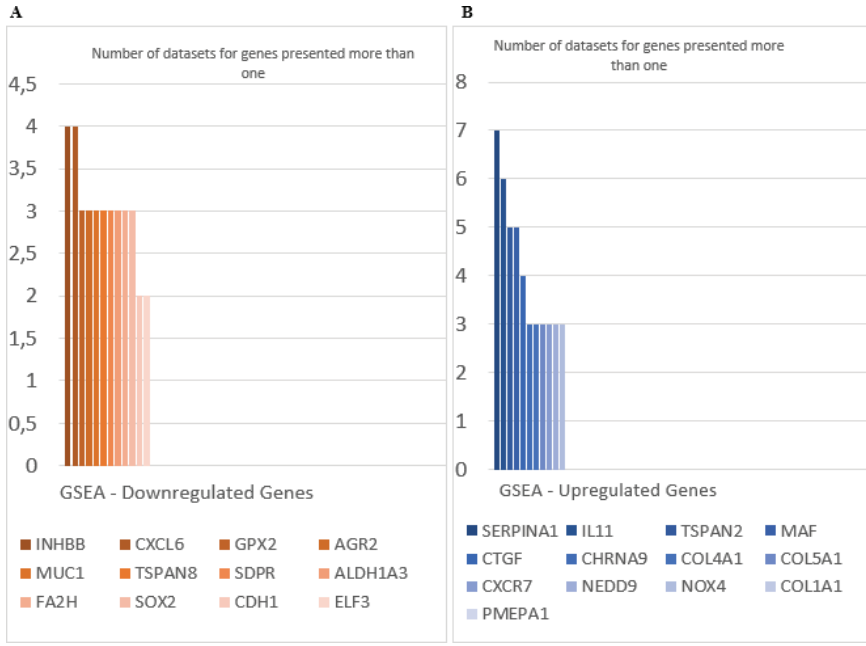
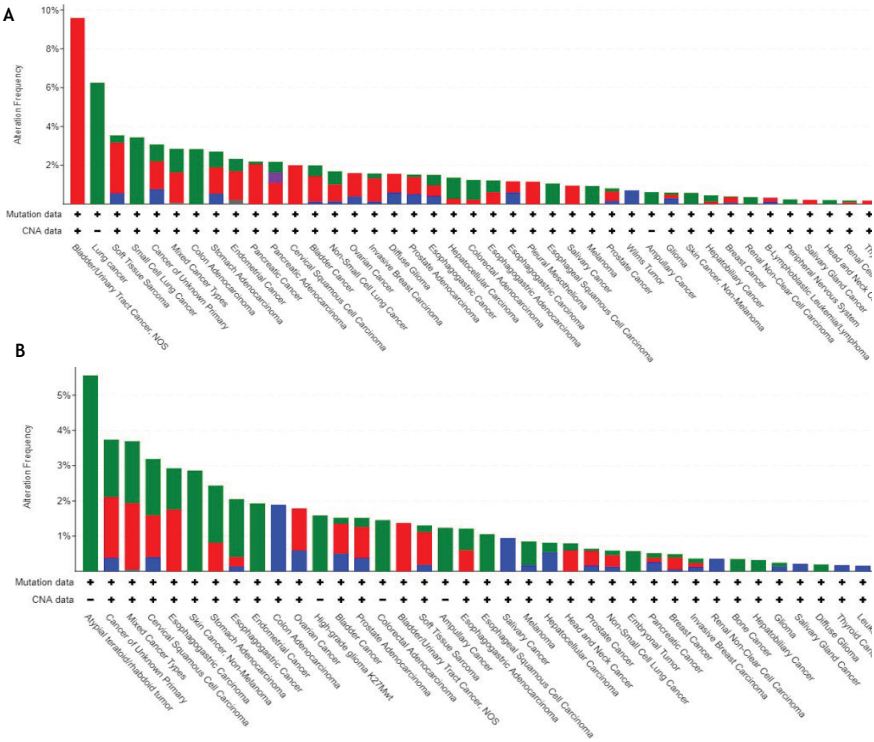


Figure 2: Gene set enrichment analysis; Differentially expressed genes common in more than one dataset in the TGFβ phenotype. (A) Downregulated genes. (B) Upregulated genes.



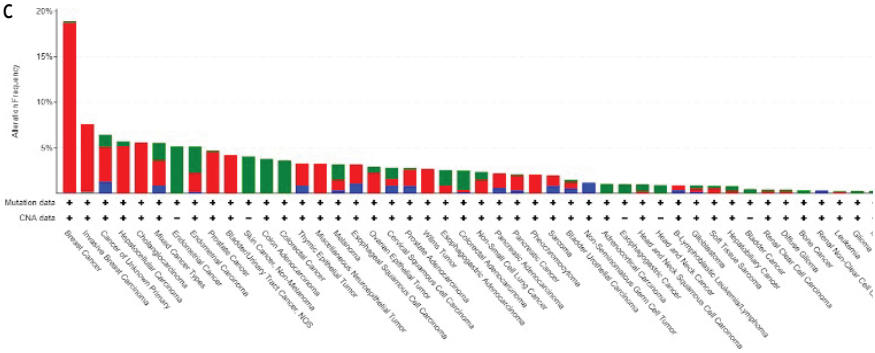


Figure 3: TGFβ gene alterations of cancer types. Y axis represents the percentages of alteration frequencies. X axis shows the cancer types. Color coding indicates the mutation type; red: amplification, green: mutation, blue: deletion, purple: fusion. (A)TGFβ1 (B) TGFβ1 (C)TGFβ3.

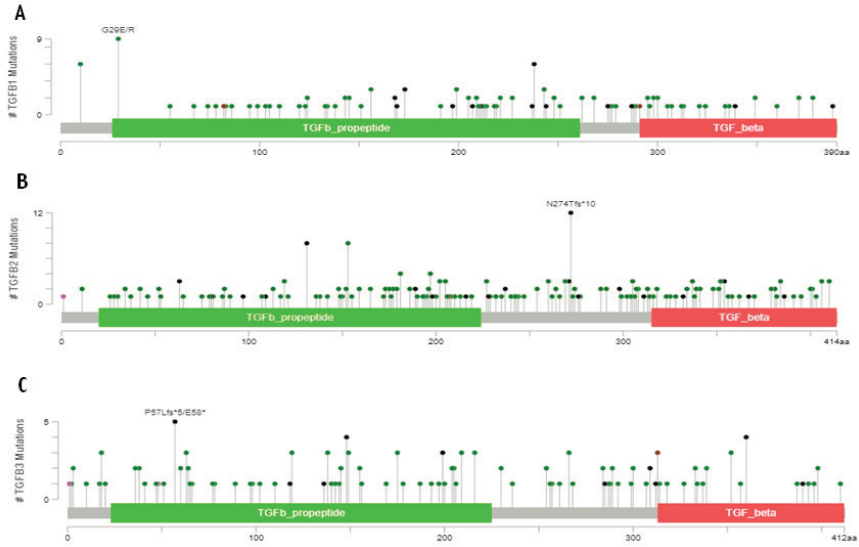


Figure 4: Number and amino acid points of the mutations in the TGFβ gene. The highest number of changes in the amino acid is shown. (A)TGFβ1, (B)TGFβ2, (C) TGFβ3.

Conclusion

EMT is involved in plenty of processes from development to wound healing and cancer metastasis (Nieto et al., 2016). Since the TGFβ molecule is the key regulator of this transition, due to its central role in many processes and therapeutic possibilities, a large amount of data already exists in the literature about TGFβ and EMT. However, studies in the literature are mainly carried out with only one isoform of the

TGFβ. Suppose the comparison among the isoforms is the case; it is only investigated in a cell line of interest like breast cancer (Hachim et al., 2018) or in a specific topic like gonadal development (Memon et al., 2008). Thus, there is a lack in the literature of a broad range of research on TGFβ isoforms and their transcriptional effects on many aspects, including datasets from different cell lines and different organisms. As indicated in this thesis's aim, differences among the isoforms of such a critical molecule are noteworthy for future research and therapeutic approaches.

To evaluate the research questions in a broad spectrum, 15 public datasets from 2 organisms of different ranges were analyzed with Bioconductor, R programming language, and a variety of online research tools (GO, GSEA). Results are consistent with the hypothesis; the differential expression and gene set enrichment analysis results indicate a significant difference in transcriptional level between TGFβ isoforms. Withal, there are common genes and functions among all three isoforms, some regardless of the organism. Mutations on a particular isotype of TGFβ are upfront in some cancer types; they differ in molecular functions; they reside in different cellular components and are associated with other pathways in some processes. The data contributes to a better understanding of the TGFβ isoforms and their transcriptional effects.

Thirteen genes were shared among all three isoforms of intersected human and mouse differentially expressed genes. These genes can be considered as TGFβ induced EMT markers as they do not depend on isoform, organism, and tumorigenic or normal cell type. Whereas genes selected for the query were specific for each phenotype, similar results in the GO analysis may show that different genes can be correlated with the same biological process or molecular function. Numbers in the intersected Venn schemes are now worthy of attention with the biological processes they participate in, molecular functions they regulate, and cellular components they reside in.

As in further research, these results can be considered when planning a target-oriented new research on TGFβ and EMT. They may affect the choice of isoform in practice. Common genes of all TGFβ isotypes from differential expression analysis results can be considered as TGFβ induced EMT markers. It can lead to a more specific way for possible drug targets and predictive markers. Especially in cancer research, since TGFβ has a dual role in tumor suppression and tumor invasion, different therapeutic approaches can develop for early and late-stage cancers based on the possible future research that may be carried out with this research's findings.

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Chapter 9

PROSTHETIC STOMATITIS

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Introduction

Denture stomatitis is defined as local or general chronic inflammation of the mucosa with which the denture base comes into contact. Clinically, redness and burning sensation are the most prominent symptoms. more rarely, it is characterized by petechiae, and it may rarely show vesicular symptoms.[1-4]

In histological examination, proliferative and degenerative changes are observed with decreased keratinization and thinned epithelial layer in the tissues.[2]

Prosthetic stomatitis cases are generally asymptomatic, but complaints such as burning, change in taste, halitosis, mucosal bleeding and dry mouth have been reported rarely.[2,3]

Denture stomatitis of multifactorial origin is the most common mucosal lesion in individuals using full dentures. It is expressed with different names. In this sense, denture stomatitis; can be named as prosthetic mouth pain, inflammatory papillary hyperplasia and chronic atrophic candidiosis.[3-5]

Factors causing denture stomatitis; [5,6]

1. Change of oral microflora,
2. Candida albicans infection,
3. Mechanical irritation of tissues due to incompatibility of the prosthesis,
4. Relief areas in the upper prosthesis,
5. Always wearing the prosthesis,
6. Inadequate oral care and inadequate denture cleaning,
7. Systemic diseases,
8. Immunological responses.

Oral microflora plays a key role in the development of stomatitis. Therefore, it is necessary to have detailed information about oral microflora. Many factors play a role in the formation of oral microflora. Some of those; age of the individual, oral hygiene, existing additional disease, teeth eruption, missing teeth, prosthesis use.[1-5]

Prostheses in the mouth prepare a suitable ground for microorganisms to settle. Prostheses can cause various levels of damage to the tissue in the area where they are placed by mechanical effects. [5-7] Acrylic resins used in the production of prosthetic base plates, although they have been

prepared with technological methods, are no longer monomers. This monomer irritate the surrounding tissue by removing them. Food residues and microorganisms belonging to the oral flora settle in the tissues that are injured for different reasons and initiate various inflammatory events. This situation can progress and become a clinical problem. In patients with prosthetic stomatitis, the use of the prosthesis is also difficult.[6,7]

Prosthetic Stomatitis Classification

Denture stomatitis can spread to different regions with different microorganisms.[5] Newton classified prosthetic stomatitis into three categories.[1] These;

Type 1: Hyperemic foci in the form of dots, local inflammation,

Type 2: Widespread hyperemia, widespread inflammation in the area covered by the prosthesis,

Type 3: Papillary hyperplasia with widespread inflammation.

Type 1 prosthetic stomatitis, which is seen as very small bleeding foci under the prosthetic base plate, is usually prosthetic-related trauma; Type 2, which is characterized by widespread hyperemia within the prosthesis margins, is associated with Candida infection. It is thought that the combination of trauma and Candida infection is effective in Type 3 prosthetic stomatitis in which diffuse hyperemia is accompanied by mucosal hyperplasia.[1] It has been reported that Type 2 stomatitis, which is not treated for a long time, may progress to Type 3.[3]

The reasons for the emergence of these types in Newton's classification also differ. Type I prosthetic stomatitis is usually due to traumatic causes. Type II and III prosthetic stomatitis occurs with the accumulation of plaque on the surface of the prosthesis and on the mucosa under the prosthesis.[8,9]

Etiology of Denture Stomatitis

Prosthetic stomatitis etiological factors are divided into two groups. These are prosthesis-related factors and infective factors. Incompatible prostheses, Insufficient prosthesis cleaning, Insufficient oral hygiene, Causing trauma in the mucosal contact areas of the prosthesis are factors associated with the prosthesis.

The presence of the prosthesis is a sufficient cause for prosthetic stomatitis due to Candida species. Infection may develop over time in individuals who wear the prosthesis constantly. This infection situation disappears when the prosthesis is removed for a while. Various bacteria can also be isolated in the infected area. [10] In areas where the prosthesis touches the mucosa, the transformation of palate epithelial cells is

stimulated due to traumatic causes and the barrier function and the degree of keratinization of the epithelium are reduced. This helps the bacterial and fungal antigens to pass into the tissue. [8,11]

Yeast growth is facilitated in patients who have poor oral hygiene, are fed with intensive carbohydrate, whose saliva flow decreases, and who constantly use the prosthesis. In this case, the pathogenicity of the prosthetic plate increases.[12]

Candida-associated denture stomatitis occurs in several stages. After microorganism colonization, adhesion occurs to the hard surfaces of acrylic resin or denture lining materials. Adherent cells then emerge. These cells are seen by the cosme of other cells in the environment. With the increase in the number of candida on the prosthetic surface, the production of acid also increases. This situation is directly toxic. Acid proteinase and phospholipase produced by Candida increase their adhesion to the surface. The progression of microorganisms in the prosthetic plates to the respiratory system or digestive system creates a risk for various infections in patients with a low immune system. [13,14]

Prosthetic Stomatitis Treatment

Since the etiology of prosthetic stomatitis is multifactorial, many different methods are used in its treatment; Many different methods such as the use of antifungal agents, prosthesis disinfection, rehabilitation or renewal of the existing prosthesis, use of soft lining materials, use of antiseptic mouthwash are among the treatment options. [15,16]

Antifungal agents can be used systemically or topically. The effectiveness of the antifungal agent depends on its concentration and the susceptibility of the disease agent Candida genus.

Nystatin, amphotericin-B, mycostatin and hexetidine are commonly used topical agents. Micostatin binds to ergester, impairing the permeability of the cell membrane and causing cell death. Amphotericin-B and nystatin prevent the adhesion of Candida to buccal epithelial cells and inhibit germ tube formation.

Nystatin and amphotericin-B taste bad, causing patients to complain. Mycostatin and hexetidin are used in the form of mouthwash. [15,17]

Denture disinfection is very important in stomatitis treatment. It has also been reported that the formation of stomatitis is related to the Candida biofilm on the acrylic base instead of the biofilm on the palatinal mucosa, however, Candida in the plaque on the impression surface of the prosthesis produce more toxins than those colonized in the mucosa.[18,19] For this reason, in stomatitis treatment, especially in cases where the number of

Candida has increased, it is recommended that the treatment be directed to the prosthesis and to ensure the hygiene of the prosthesis. Brushing alone is insufficient for the control of prosthetic biofilm. Mechanical cleaning should be supported with chemical agents.[3,19]

Chlorhexidine is an antiseptic and disinfectant agent. It is effective against bacteria, viruses and fungi. It shows a pronounced effect on the target cell depending on its concentration. At low concentrations, bacteria and fungi attach to the cell wall and cause the structure of the membrane to deteriorate. In high concentrations, it affects the cytoplasmic components. Can be used as mouthwash, topical gel or dip solution. [20,21]

Mechanical Cleaning

Using soap or toothpaste with a brush is the most common method of cleaning dentures. It has been reported to be a highly effective method against stains and plaque on the prosthesis.[22] Twice a day brushing with toothpaste has been reported to be effective, but brushing with soap has also been shown to be highly effective on plaque. Although the use of toothpaste is effective in removing microorganisms, the abrasive effect of the paste causes roughness on the prosthesis surface and abrasion on the base material and artificial teeth.[22,23]

The use of ultrasonic devices is also one of the mechanical cleaning methods. However, its use alone is not sufficient for disinfection. If a disinfectant solution is put into ultrasonic devices, the effectiveness of the disinfectant can be increased and a significant reduction in the number of microorganisms can be achieved.

Microwave ovens are tools that can be used for cleaning prostheses. It has been known that prostheses infected with C.albicans are sterile in 6 minutes in a kitchen-type microwave oven (2450 MHz, 350 W). This method provides a more effective sterilization than keeping the prostheses in a solution of 0.02% and 0.0125% sodium hypochlorite for 8 hours.[22]

Chemical Cleaning

Disinfectants, 0.4 and 1% potassium permanganate solution, 2% glutaraldehyde solution, chlorine dioxide and chlorhexidine gluconate solutions are also used in the disinfection of removable dentures. It has been reported that a 2% glutaraldehyde solution provides disinfection for 10 minutes.[22,24]

Enzyme-containing cleansers act by breaking down glycoprotein, mucoprotein and mucopolysaccharides in plaque. They are effective on organic deposits on the prosthesis, but EDTA must be added in order to

affect inorganic substances. Enzymes such as papain, mutease, protease and amylase are included in cleaning solutions.[22]

They contain alkaline hypochlorites, sodium hypochlorite. They show bactericidal and fungicidal effects by dissolving the organic structure.[25] Sodium hypochlorite is effective on many microorganisms, including spores. It has been reported that holding prostheses in 5.25% solution for 5 minutes is the most effective bactericidal and fungicidal method. They are in the form of alkaline peroxides, powder or effervascent tablets. Peroxide cleaners are most effective on newly formed plaque and stains.[26]

There are many studies arguing that the most important factor in the etiology of prosthetic stomatitis is trauma, so treatment should eliminate trauma. [2,4,27] Prevention of prosthesis-related trauma may be in the form of improvement or replacement of incompatible prostheses, or it can be applied by reducing the pressure on the tissue by using soft lining materials.

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Chapter 10

LIMBIC SYSTEM

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The "limbic" definition of the limbic system, also defined as the emotional brain (Julian et al., 2015:55), was made by Paul Pierre Broca in 1878. In 1937, American doctor James Papez, after injecting the rabies virus into the hippocampus of a cat and monitoring its distribution in the brain, first published an article describing the specific brain circuits in detail and defined the "Papez circuit" in this neuroanatomical model (Figure 1) (Rajmohan and Mohandas, 2007:49).

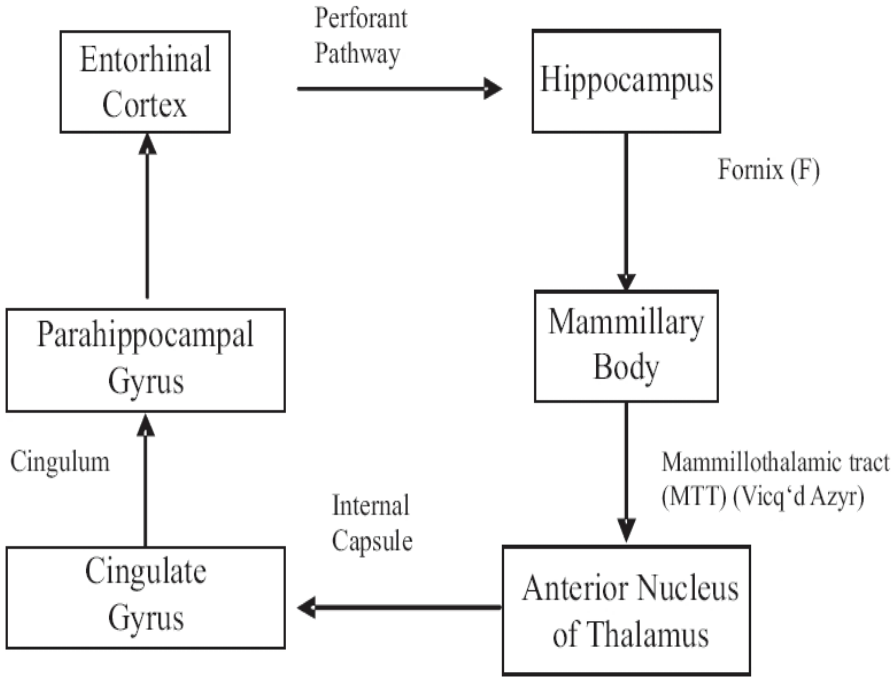

Figure 1. *Papez circuit (Rajmohan and Mohandas, 2007:49).*

Defining the Papez circuit as an elementary circuit has made it easier for us to understand its functionality. The content of this circuit is that the emotion realized through the hypothalamus is controlled and modulated by the fibers coming from the fornix. In addition, it is understood that cortical control of emotional activity is a pathway originating from the cingulate gyrus. Figure 2 shows the afferent and efferent connections of the hippocampal formation semidiagrammatically (Willis and D.E. Haines, 2018:31).

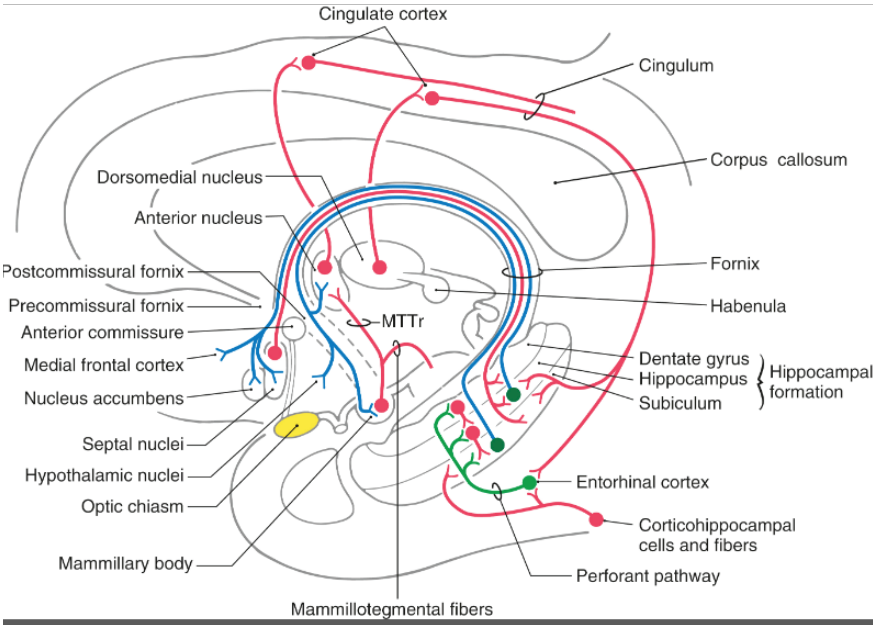

Figure 2. *Afferent and efferent connections of the hippocampal formation. (MTTr, mamillothalamic pathway) (Willis and D.E. Haines, 2018:31).*

In 1948, the neuronal circuit called "Yakovlev circuit" was defined. It is stated that the neuroanatomical connections here are between orbitofrontal, temporal, amygdaloid and thalamic regions. In Yakovlev's definition, the relationship between emotions and the limbic system has been revealed more (Takeda et al., 2007:47). Paul MacLean is known in history for finding the term "limbic system" in 1952 by formulating and elaborating on the limbic system concept (Paul D. MacLean, 1999:2). Figure 3 shows the structures that forming the limbic lobe (Purves et al., 2001:2). Subsequently, the connection of other components of this system with the hypothalamus was emphasized (Nauta W, 1958:81; White et al., 2008:34).

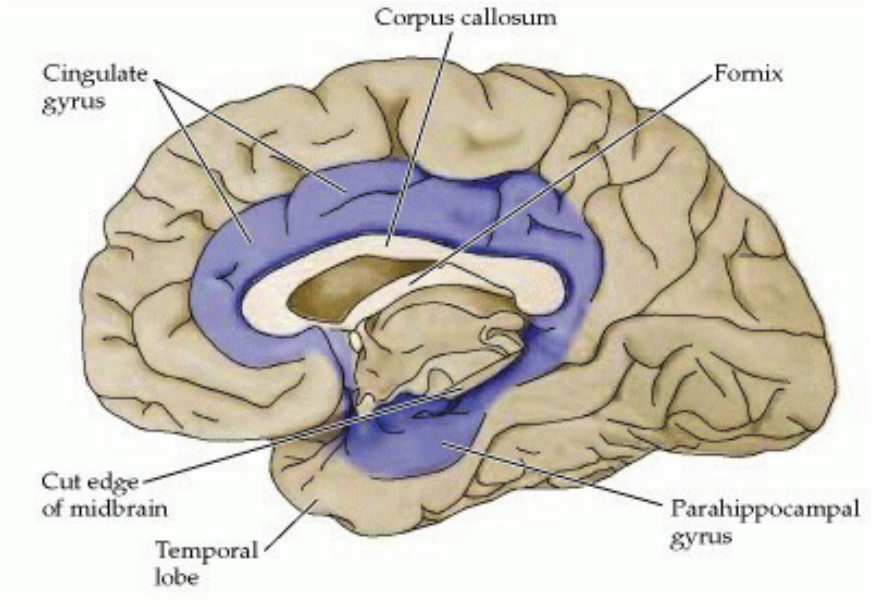

Figure 3. *Limbic lobe (Purves et al., 2001:2)*

The limbic system is a structure that has complex networks in controlling and/or processing emotions, with connections to memory, learning, sexual behavior, perception of smell, feeding behavior, fear, aggression, social behavior, defense and motivation processes. (Sokolowski and Corbin, 2012:5, Bennett at eal., 2015:25, Torrico and Abdijadid, 2021).

In the anteromedial temporal lobe of the limbic system, areas such as the amygdala, the hippocampal complex and the entorhinal cortex and the cingulate cortex are connected via the thalamus (especially the anterior thalamic and medial dorsal nuclear groups as well as the midline thalamic nuclear group) and projection into the hypothalamus, midbrain and anteromedial its projection to the temporal lobe is mentioned in the literatüre (Jo et al., 2019: 10). It was observed that the Klüver-Bucy syndrome, which was first reported in 1939 in monkeys with bilateral temporal lobectomy, developed similarly in humans (with herpes simplex encephalitis, bitemporal damage from surgical ablation, and dementing conditions such as Pick disease). In animals where this syndrome develops, inappropriate mating behavior with objects and eating, fearless behaviors when encountering an enemy are observed, while behavioral changes such as visual agnosia, prosopagnosia, changes in sexual preference, memory loss, calmness, binge eating occur in humans (Thompson and Umphred, 2020: 4).

The limbic system does not only interact with physiological processes, but also plays an active role in pathologies such as mesial temporal lobe epilepsy, which is one of the most resistant adult forms of epilepsy, and the hippocampus is a structure that causes seizures in the temporal lobe epilepsy. (Behr et al., 2014:114). Given the role of the hippocampus in memory, it is usual to expect short-term memory problems in any situation where the hippocampus is affected (Lövblad et al., 20147:35). It has also been associated with obsessive-compulsive disorder, autism, schizophrenia, Alzheimer's disease, major depressive disorder. Structural changes in limbic pathways have been found in these pathologies (Yu et al., 2014:6).

It has been found that in autism, which is a severe neurodevelopmental disorder, the amygdala changes depending on age. These changes include volumetric differences of the amygdala, abnormal amygdala activity and social cognition, and amygdala damage. There is growth in the amygdala, especially in early childhood (Schumann et al., 2009: 66; Hennessey et al., 2018: 90), but this growth slows down or reverses at 8-14 years and older (Barnea-Goraly et al., 2014: 48; Hennessey et al., 2018: 90) in the literature, the amygdala volumes are normalized in older adolescents and adult individuals with autism. The increase in amygdala volume is also thought to be related to the severity of social symptoms (Schumann et al., 2009: 66). It has been reported that these areas, which include the amygdala as well as the cingulate gyrus, septum, and hypothalamus, which are included in the neurological networks associated with autism, may be associated with dysfunctions such as emotion, impulse, influence, fear, and aggression in autism (Wendy et al.2012: 59).

Early life stressors have been shown to exacerbate epileptogenesis in limbic epilepsies and mediate some limbic modulation. It has been found that both prerenal and postnatal stress increase the occurrence of seizures in various experimental models of epilepsy. Similarly, early life stresses are known to cause changes in limbic circuits and disrupt long-term potentiation in the CA1 region of the hippocampus (Koe et al., 2009: 3). It has been demonstrated that the decrease in functional connectivity between limbic areas in patients with mesial temporal lobe epilepsy may be related to cognitive and/or behavioral, emotional and memory-related limbic pathologies seen in patients with mesial temporal lobe epilepsy (Jo et al., 2019: 10). The basis of these studies is based on an article published in 1957, which reported that despite the recovery of epilepsy as a result of surgical excision of the hippocampus and hippocampal gyrus of a patient with epilepsy, permanent memory loss occurred. This person was unable to create new memories (Scoville and Milner., 1957: 20). To summarize, it has been proven in many studies that limbic structures such

as the entorhinal cortex, thalamus, hippocampus, and amygdala can play an active role in the pathogenesis of epilepsy.

It has been suggested that changes in limbic structures in Parkinson's disease can be demonstrated by multimodal magnetic resonance imaging. These changes may be associated with non-motor symptoms and daily living activities. The active role of the amygdala and hippocampus in the neuropathology of Parkinson's has been emphasized in recent studies (Ernest et al., 2019: 76). The findings of a study investigating the differential diagnosis level of magnetic resonance imaging and volumetric analysis in the amygdala and its lower segments in cognitive impairment in Parkinson's disease have shown that bilateral corticoamigdaloid transition area volumes can predict cognitive impairment in Parkinson's disease to a great extent. The bilateral corticoamigdaloid transition area plays a critical role in mental impairment continuity. et al., 2019: 11). In another study, it was found that there are changes in limbic gray matter in the limbic system in Parkinson's disease depending on age (Li et al., 2017: 38).

It is known that the limbic system plays a role in the pathogenesis of schizophrenia. The limbic system plays an active role in the development of symptoms seen in patients with schizophrenia, especially hallucinations and external interpretation such as intrinsic and delusions. In addition to being a structure responsible for the initial formation of declarative memories, the hippocampus is sensitive to stress, causing it to restrict the responses to biological stress through the hypothalamic-pituitary-adrenal system. It has been reported that the parahippocampus, which takes widespread input from the cortex and integrates multimodal information, is involved in the formation of spatial memories (White et al., 2008: 34). In schizophrenia, which is characterized by impaired social interaction, it has been suggested that the severity of delusion and functional changes in the limbic system are related to the misinterpretation of gestures by delusional patients (Bucci et al., 2008: 158; Stegmayer et al., 2018: 44). When the structures of the limbic system are examined in post-mortem studies in individuals with schizophrenia, the number of neurons in the cingulate cortex (Benes et al., 1986: 43), a decrease in the number of interneurons (Benes et al., 1991: 48) and also the parahippocampal gyrus (Brown et al., 1986: 43 Altshuler et al., 1990: 47) and the amygdala (Bogerts et al., 1985: 42) were found to be reduced in volume (White et al., 2008: 34).

The development process of the processes associated with the limbic system is understood from lesion studies or cytotoxic damage models. The information obtained from lesion studies is difficult to distinguish between nuclei, as many inputs are intertwined due to the structure of the

limbic system. While some of these structures are innate features, some are gained by reinforcing and learning with experience. Inborn behaviors are the characteristics that ensure the survival of the individual and the continuity of his lineage. (Sokolowski and Corbin, 2012: 5). Central nervous system disorders such as brain injury, cerebrovascular accident, tumors, inflammatory disorders, Alzheimer disease, Parkinson disease, and vestibular disorder are closely related to the limbic system (Thompson and Umphred, 2020: 4)

The limbic system is formed by structures located between the upper part of the brainstem and the forebrain lateral to the thalamus (Torrico and Abdijadid, 2021). The limbic system consists of the limbic cortex, hippocampal formation, amygdala, septal area, and hypothalamus. While the brain regions forming the limbic cortex are the cingulate gyrus and parahippocampal gyrus, hippocampal formation forms the dentate gyrus, hippocampus and subicular cortex (Rajmohan and Mohandas, 2007: 49).

Located at the intersection of many neural pathways, the hypothalamus is the main control center of the limbic system. The limbic cortex refers to the two C-shaped gyri surrounding the corpus callosum, including the cingulate gyrus, parahippocampal gyrus and olfactory lobe.

In Figure 4, limbic networks within limbic structures are shown. There are various neurotransmitters in the limbic system such as dopamine, serotonin, acetylcholine, and norepinephrine (Thompson and Umphred, 2020: 4).

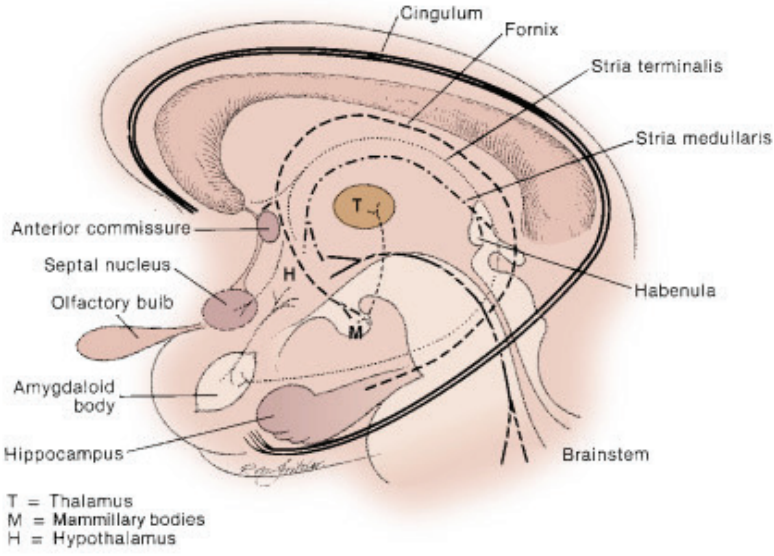

Figure 4. *Neuron Network Within the Limbic System (Thompson and Umphred, 2020: 4)*

There are numerous nuclei (Table 1) in the hypothalamus, whose front-to-back location is divided into the supraoptic region, the tuberal region and the mammillary region (Rajmohan and Mohandas, 2007: 49). The hypothalamus is a structure located opposite the third ventricle, in the sagittal plane, it divides into a supraoptic region containing three nuclei, an intermediate (tuberal) region with five nuclei, and a posterior (mamillary) region with three nuclei, while in the coronal plane it is divided into lateral, medial and periventricular regions (Mtui et al., 2021: 34).

Table 1. *The hypothalamic nuclei (Rajmohan and Mohandas, 2007: 49).*

Region	Medial area	Lateral area
Supraoptic	Supraoptic nucleus	Lateral nucleus
	Paraventricular nucleus	Part of Supraoptic nucleus
	Anterior nucleus	
	Suprachiasmatic nucleus	
Tuberal	Dorsomedial nucleus	Lateral nucleus
	Ventomedial nucleus	Lateral tuberal nuclei
	Arcuate nucleus	
Mamillary	Mamillary body Posterior nucleus	Lateral nucleus

As a structure of the limbic system, the main functions of the hypothalamus include the regulation of growth and metabolism, regulation of the endocrine system, regulation of fluid balance, control of food intake, attack and defense, regulation of body temperature, sleep-wake cycle and memory, and mental events (Mtui et al., 2021: 34).

The amygdala, which is an almond-shaped structure deep in the temporal lobe, consists of approximately 13 nuclei but can be divided into 5 main groups (basolateral nuclei, cortical-like nuclei, centromedial nuclei, other amygdaloid nuclei, extended amygdala (centromedial amygdala, sublenticular substantia innleta and the bed) stria terminalis) and its lower parts (Rajmohan and Mohandas, 2007: 49). There are two main outflow paths (Figure 5): the dorsal pathway of the amygdala to the septal area and the hypothalamus, and the ventral pathway to the septal area, the hypothalamus and the medial dorsal thalamic nucleus (Rajmohan and Mohandas, 2007: 49).

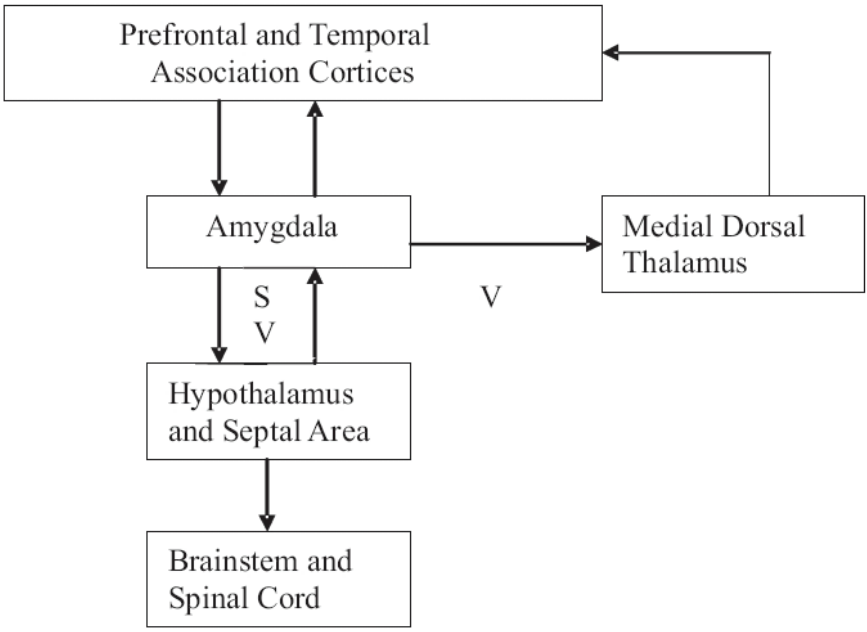

Figure 5. *The amygdala major output pathways (Amygdalo-septal pathway, S: Stria terminalis, V: Ventral amygdalofugal pathway) (Rajmohan and Mohandas, 2007: 49).*

Amygdala; through connections with the hypothalamus in the regulation of emotional responses by interacting with the autonomic and

endocrine system, its links with the hippocampus are also effective in the formation of memories (Morgane et al., 2005: 75; White et al., 2008: 34).

It has been shown in animal studies that amygdala lesions impair the development of conditioned fear and induce docility by leading to marked reductions in anxiety and aggression. Similarly, activation of the amygdala in response to fear and anger has been proven in functional imaging studies in humans (Zald and Pardo, 1997: 94, Whalen et al., 2001: 1, White et al., 2008: 34). The close connection of the main components of the olfactory pathways between the limbic areas (amygdala and hippocampus) is shown in Figure 6 (Mouly and Sullivan., 2010: 15).

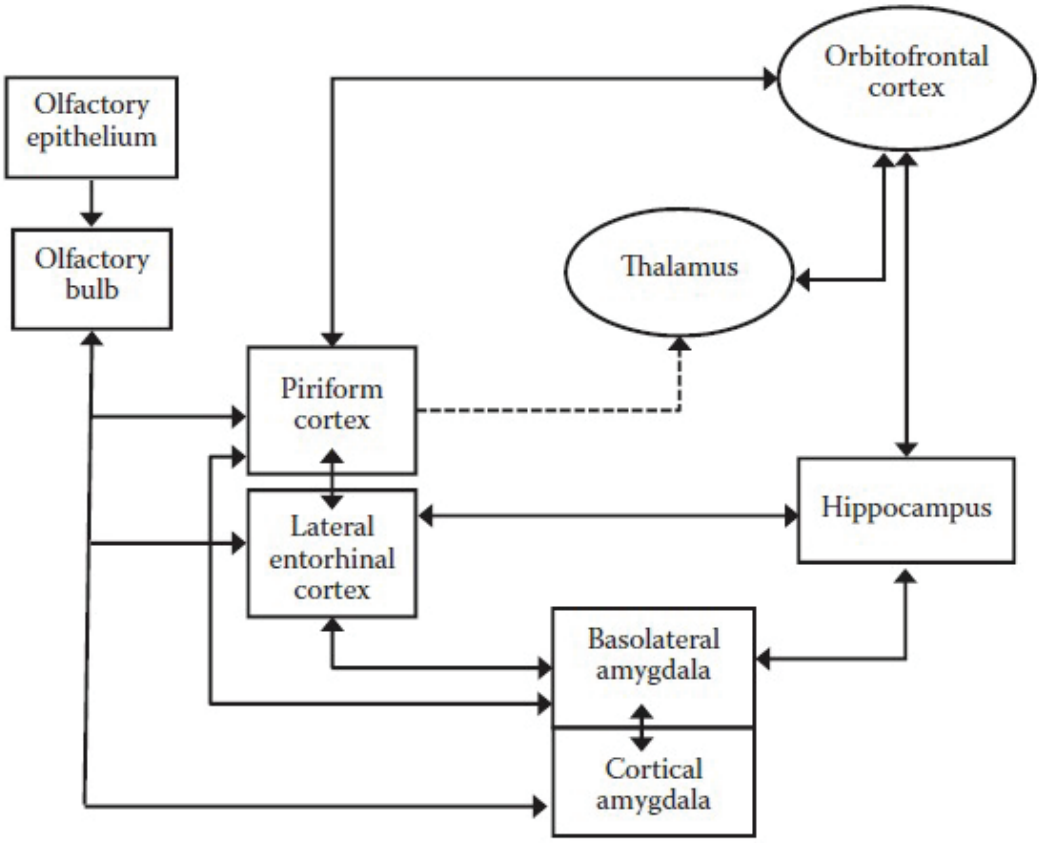

Figure 6. *Schematic representation of the main components of the olfactory pathways with limbic areas (amygdala and hippocampus) (Mouly and Sullivan., 2010: 15).*

The movement (MOVE; the M (motivation, Memory); the O (olfaction); the V (visceral); and E (emotional)) defined by Moore in 1932 by combining the initials of the limbic system of functionality. It is summarized in Figure 7 (Thompson and Umphred, 2020: 4).

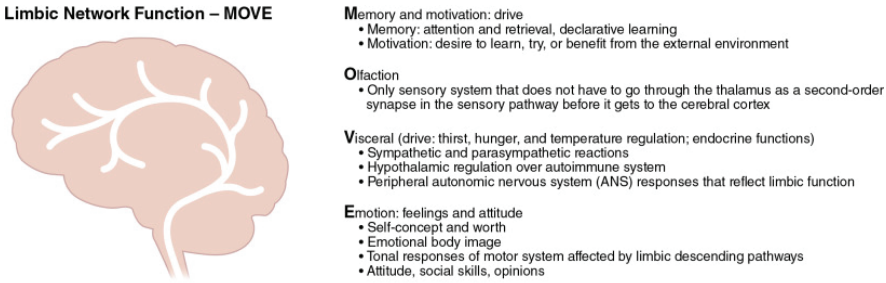

Figure 7. *Relationship of the limbic system with motion (Thompson and Umphred, 2020: 4).*

To summarize the functions of limbic system structures and connections, the cingulate gyrus is responsible for autonomic functions regulating heart rate and blood pressure as well as cognitive, attentional and emotional processing. Parahippocampal gyrus is active in spatial memory and the hippocampus in Long-term Memory. Anxiety, aggression, fear conditioning; The amygdala is related to emotional memory and social cognition. It is also associated with memory in the Mammailary body. Hypothalamus regulates the autonomic nervous system via hormone production and release. Secondarily affects and regulates blood pressure, heart rate, hunger, thirst, sexual arousal and the circadian rhythm sleep/wake cycle. Nucleus accumbens responsible for reward, addiction (Rajmohan and Mohandas, 2007: 49).

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Chapter 11

FOODBORNE VIRAL INFECTIONS AND DETECTION METHODS

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Introduction

Food contamination through microbial factors is a significant global health problem. In addition to bacterial food infections, viral food infections whose importance is more recognized recently pose a grave challenge to public health and also to national economy. Centers for Disease Control and Prevention (CDC) informs that more than 250 foodborne diseases are sourced from bacteria, viruses, parasites, protozoon, mold, etc. microbial pathogens (CDC 2018). World Organization of Health (WHO) notes that one in every 10 people (~ 600 million) suffers from health problems related to food sourced diseases due to consumption of contaminated food and nearly 420,000 people die every year due to this condition. It also declared that 30% of cases are children under age 5. According to data from the WHO, when 3 million children younger than age 5 are added, the estimated cases of foodborne diseases in Europe is 23 million. WHO declared the number of such cases in Africa is more than 91 million including 137,000 deaths (WHO 2015). According to data provided by the CDC, every year 48 million diseases are reported in the U.S. due to foodborne causes and 9.4 million of these cases are sourced from well-known pathogen microorganisms (CDC 2018). It is estimated that annually around 685 million cases are affected from gastroenteritis based on globally effective norovirus, causing 200,000 deaths (Pal & Ayele, 2020).

Viruses are microorganisms that do not have cellular structures, can have only one type of nucleic acid (RNA or DNA) covered with protein layer or capsule, have diameters of 25- 40 mm and small structures, that can develop and multiply in living cells that are named known as host cells (Erol, 2007). Viruses have their own genomes separate from host cell genome. However, because they do not have cellular structures, they do not have original metabolisms. They are dependent on host cells for living cell activities such as energy, metabolic by products, and protein synthesis which makes them obligatory intracellular parasites (Martinko et al., 2016).

Contamination of food with viruses take place in 2 ways being direct (primary) or indirect (secondary):

1. Direct (Primary) Contamination: This mode of transmission is sourced from milking, slaughtering, pre-harvest stages based on farm to table or field to fork principle (vegetables-fruits produced with contaminated fecal water and fertilizers, milk and meat of infected animals, shellfishes collected in contaminated waters, etc.).

2. Indirect (Secondary) Contamination: Viral food infections from humans and animals take place indirectly. This takes place at processing, storing, distribution, etc. stages of food (contaminated milk, pastry products, salads, shellfish, etc.). This is most effective when employees working at ready food sale points such as restaurants, cafes do not follow hygiene criteria or use tools and materials contaminated with virus (Erol, 2007; Ray & Bhunia, 2016; Pal & Ayele, 2020). Most of foodborne diseases zoonotic in nature. One of the main factors in zoonotic diseases whereby viruses infect people is consumption of contaminated animal origin products or infection while processing by employees in charge of production of products infected with target factor. Another reason is contact of food with water contaminated with excrement.

According to studies conducted in developed countries in the entire world, Norwalk and the such viruses (Norovirus) and viral infections sourcing from Hepatitis A cause the main reason of viral gastroenteritis and are listed in top 10 causes of foodborne epidemics (Pal & Ayele, 2020; Koopmans & Duizer, 2004). Viruses such as Hepatitis A, Norovirus, Rotavirus, Tick-borne encephalitis, Poliovirus, Echovirus, and Astrovirus are among the most important types (Muratoğlu et al., 2015). Foodborne viral infections are examined in 2 classes as viral gastroenteritis and viral hepatitis. There are difficulties with detection of enteric viruses because they cannot multiply in food and exist in minimal amounts (Erol, 2007).

Viruses that can infect through contaminated food target tissues for infection, meaning gastrointestinal system (enterotropic), liver (hepatotropic), nervous system (neurotropic) or the respiratory system (pneumotropic). Among enterotropic viruses there are NoV, rotaviruses, sapoviruses, enteric adenoviruses among others. Hepatitis A (HAV) and Hepatites E viruses (HEV) are hepatotropic, while human enteroviruses and some other main viruses are neurotropic. Viruses that cause SARS and bird flu cause pneumotropic disease. Viral gastroenteritis frequently causes vomiting and diarrhea. Foodborne viruses that are transmitted and that may be related to all these described are presented in (Table 1) (5, 6) (Bosch et al., 2016; Jaykus & Escudero, 2018).

Food and waterborne pathogen viruses are separated into 3 categories.

1. Viruses that cause gastroenteritis: Astrovirus, Rotavirus (group A-C), Enteric Adenoviruses and two Enteric Calicivirus types, meaning small structured and round viruses (Small Structure Viruses=SRSV) or ‘Norwalk Like Viruses’ (NLV) and typical Caliciviruses or ‘Sapporo Like Viruses’ (SLV), Coronaviruses

2. Fecal-orally transmitted hepatitis viruses: Hepatitis A virus (HAV), Hepatitis E virus (HEV),

3. Viruses causing different nervous symptoms and other conditions: Enterovirus, polioviruses, etc.

(At sever et al., 2015; Koopmans et al., 2002).

Another method in classifying viruses is based on their nucleic acid being RNA or DNA, their symmetry, being in a sheath or not, diameter of nucleocapsids and sequence of nucleic acid (Öksüztepe & Demir, 2016; Yörük, 2021).

Table 1. *Viruses that may be foodborne transmitted*

Virus	Genus	Family	Genome	Common Diseases	Transmissions
Human Norovirus	Norovirus	*Caliciviridaes*	sRNA	Gastroenteritis	Oral-fecal route, PtP contact, aerosol, Foodborne (FBO), Waterborne (WBO)
Human Sapovirus	Sapovirus	*Caliciviridaes*	sRNA	Gastroenteritis	Oral-fecal route, PtP contact, aerosol, FBO, WBO
Hepevirus	Ortohepevirus	*Hepeviridae*	ssRNA	Acute Hepatitis	Waterborne (WBO)
Aichivirus	Kobuvirus	Picornaviridaes	sRNA	Gastroenteritis	Oral-fecal route FBO, WBO
Human Astrovirus	Mamastrovirus	*Astroviridaes*	sRNA	Gastroenteritis	Oral-fecal route, PtP contact, aerosol, FBO
Human Parechovirus	Parechovirus	*Picornaviridaes*	ssRNA	Respiratory and gastrointestinal symptoms in young children, with occasional infection of the central nervous system	FBO
Echovirus	Enterovirus	*Picornaviridaes*	sRNA	Gastroenteritis meningitis, flaccid paralysis	Recreational waters WBO
Coxsackievirus	Enterovirus	*Picornaviridaes*	sRNA	Meningitis, herpangina cranial nerve dysfunction, pharyngitis, conjunctivitis	Recreational waters WBO
Hepatitis A virus	Hepatovirus	*Picornaviridaes*	sRNA	Hepatitis	Oral-fecal route, PtP contact, FBO, WBO
Hepatitis E virus	Hepevirus	*Hepeviridaes*	sRNA	Hepatitis	Zoonotic, Oral-fecal route, FBO, WBO
Human Picorbirnavirus	Picorbirnavirus	*Picobirnavirdae*	dsRNA	Gastroenteritis	Recreational waters WBO

Human Bocavirus	Bocavirus	*Parvoviridaes*	sDNA	Gastroenteritis	Respiratory droplets, Airborne, Oral-fecal route
Norovirus	Norovirus	*Caliciviridaes*	sRNA	Gastroenteritis	Oral-fecal route, PtP contact, aerosol, FBO, WBO
Sapovirus	Sapovirus	*Caliciviridaes*	sRNA	Gastroenteritis	Oral-fecal route, PtP contact, aerosol, FBO, WBO
Rotavirus	Rotavirus	*Reoviridae*	dsRNA	Gastroenteritis Five species (types A–E)	Oral-fecal route, WBO
Enteric SARS coronavirus	Coronavirus	*Coronaviridaes*	sRNA	Pleomorphic Respiratory, Gastroenteritis	Respiratory Droplets, PtP contact, Oral-fecal route
Ebolavirus	Ebolavirus	*Floviridae*	ssRNA	Gastroenteritis Hemorrhagic fever	Blood or body fluids, Semen from a man who recovered from EVD, Objects, Infected fruit bats or nonhuman primates
Human Enteric Adenovirus	Mastadenovirus	Adenoviridae	dsRNA	Gastroenteritis fever, respiratory disease	Respiratory droplets, Oral- fecal route WBO

Virus	Genus	Family	Genome	Common Diseases	Transmissions
Human Parvovirus	Parvovirus	*Parvoviridae*	ssRNA	Gastroenteritis	Respiratory secretions, blood and blood products
Poliovirus	Enterovirus	*Picornaviridaes*	ssRNA	Gastroenteritis flaccid paralysis, fever	Fecal-oral route, via salivary and respiratory droplets, and in some cases via conjunctival secretions and skin lesion exudates.
Nipahvirus	Henipavirus	*Paramyxoviridae*	ssRNA	Encephalitis, respiratory disease	Contaminated food or directly between people
Tick-borne Encephalitis virus	Ravivirus	*Flaviridae*	ssRNA	Encephalitis, Meningitis,	Woodland ticks, including *Ixodes scapularis*, I. ricinus and *I. persulcatus*,[9] or (rarely) through the non- pasteurized milk of infected cows
Human Coronavirus (SARS, MERS CoV)	BetaCoronavirus	*Coronoviridae*	ssRNA	Respiratory disease, SARS, MERS, Gastroenteritis	Through contacting with stool, vomitus, urine, serum and cerebrospinal fluid

Avian Influenza virus	Influenzavirus	*Ortomyxoviridae,*	ssRNA	Influenza, Respiratory Disease	Intranasal or conjunctival inoculation while swimming in contaminated water or, inhalation or ingestion of water.

**ss: single stranded*

**ds: double stranded*

Hepatitis A Virus

Hepatitis A virus (HAV) is a nonenveloped, single stranded RNA virus from *Picornaviridae* family, in *Hepatovirus* type. Its diameter is 27-32 nm. However, it could be developed in mammal cell cultures. HAV has 6 types of genotypes (I – VI). Genotypes I, II, and III include strains related to human infections and genotypes I and III are mostly held responsible. Its infective dose is about 10 and 100 virus particles (10^9 particle/g). Based on virus structure it is quite resistant to acid, heat, frosting, drying, chemicals, low levels or disinfectants such as chlorine and ozone, gamma and UV rays and can stay alive in environmental conditions for a long time and in feces for at least 1 month (Erol, 2007; Ray & Bhunia, 2016; Bintsis, 2017). HAV is inactivated when treated in 85°C and above heat, in formalin and chlorine (Atasever et al., 2015).

HAV cannot reproduce in contaminated food and must be in human body for reproduction. Because it is intestine based, contamination takes place in fecal-oral route or with human to human contact. One of the most frequent foodborne ways of contamination is shellfish (mussel, oyster, etc.) gathered from water contaminated with human feces and drinking water. Another way of contamination is consumption of food prepared by infected employee(s) (carriers are also accepted as risk factor) before cooking or without sufficient heat treatment or consumption of food contaminated after cooking (from contaminated food equipment, counters, table tops, etc.). Especially during preparation of food such as salad, sandwich, cream and such food, HAV contamination is frequent (Erol, 2007; Ray & Bhunia, 2016; Pal, 2020). Milk and dairy products also play a major role in infection with parameters (Atasever et al., 2015).

After virus is taken into body through consumption with food or with other contamination methods, some symptoms of disease are seen in humans. Virus enters into very quickly and attaches to hepatocytes and spreads in kupffer cells. At infected people symptoms of disease are shaped in around 4 weeks (Ray & Bhunia, 2016). Disease has 4 stages:

1. Viral replication (symptoms do not exist),

2. Preicteric phase: The stage when patients display symptoms such as nausea, vomiting, and fatigue,

3. Icteric phase: Dark urine with hepatomegaly, pale feces, jaundice and pain in top right side of the abdomen,

4. Recovery phase: This is the phase when symptoms improve and liver enzymes turn to normal (Jaykus & Escudero, 2018).

In acute phase of the disease loss of appetite, vomiting, diarrhea, fever, headache, fatigue, stiffness in abdomen, affection of bile ducts, bilirubin, and liver enzymes with hepatocytes and jaundice are Hepatitis findings in advanced periods. During jaundice skin, eye mucosa, feces, etc. turn pale while urine gets darker. The disease progresses for an average of 28 days in 2-4 weeks which changes based on severity of disease, babies, children, pregnant women, people with chronic health conditions, elderly, immune system conditions and virulence of the microorganism (Erol, 2007; Ray & Bhunia, 2016). Similarly, CDC informs that Hepatitis A infection is more common in people over the age of 50 and others with chronic liver conditions and in 2017 there were 6.700 HAV cases in the U.S.A. (CDC 2019). Encountering viral antigens and / or genomic materials of HAV in spleen, kidneys, tonsils, and saliva suggests possible existence of other replication regions (Koopmans et al., 2002). Researchers report that in underdeveloped and less developed countries, at areas where human population is dense, and in insufficient hygiene and sanitation conditions rate of HAV seropositivity can rise up to 95%; while this rate in developed countries is around 5-10% (Lees, 2000).

Noroviruses (Norwalk and Norwalk like Viruses)

Norwalk-Like Viruses (NLV) are named Small, Round, Structured Virus (SRSV) and called *Noroviruses* in the new classification. According to notification of European Centre for Disease Prevention and Control they are the cause of "winter-vomiting disease" or "stomach-flu" (ECDC, 2013). These viruses in *Caliciviridae* family have diameters of 32 nm, are nonenveloped and with single RNA (Koopmans et al. 2002; Erol, 2007). Their infective dose is about 10 virus particles. Noroviruses have at least 3 gene groups depending on their capsid region and RNA polymerase enzyme differences (GI-GV). Among such gene groups GI, GII, and GIV cause infection in humans, especially GII. They play role in 4 NoV infections (Ray & Bhunia, 2016; Nasheri, 2019). They are quite resistant against HAV, acid, chlorine, frosting and heating in 60°C for 30 min (Erol, 2007; Ray & Bhunia, 2016). Hand disinfectants prepared with urea and citric acid formulation are quite effective against NoV and similar viruses (Ionidis, 2016).

Studies on Norwalk started initially with an epidemic that started among school children 1968 in Norwalk town in Ohio state of the U.S.A. and the virus could be isolated (Ray & Bhunia, 2016; Pal & Ayele, 2020). However, it could not be diagnosed until 1972 when it was defined by Dr. Albert Z. Kapikian as Norwalk virus. Its current name is neurovirus (NoV) (Ray & Bhunia, 2016). The highest death rate related to NoV is seen in adults on or above 65 years of age (Hall, 2012). According to WHO, NoV is the main cause of acute gastroenteritis in children younger than 5 (WHO, 2015). CDC notifies that every year around 50.000 children in developing countries lose their lives due to NoV infections and that NoV-related infections are more common in winter months (CDC, 2017). Researchers notified that while Norovirus was responsible for acute viral gastroenteritis cases globally, in the U.S.A. it causes an estimated 5.4 million foodborne diseases annually (Bintsis, 2017).

Neurovirus is taken primarily with contact, through water droplets in the air, with food contaminated with vomit and diarrhea and infects from person to person. It is noted that neurovirus epidemics are mostly seen and might be seen at areas of mass consumption (restaurants, dormitories, military areas, ships, hotels, etc.). Bivalve shellfish such as mussels and oysters take their nourishment through filtration and collect small particles in waters such as algae and virus and accumulate them in their structures (Ghalyoun & Alçay, 2017). Frozen food, cream, and sandwich are among main sources while lettuce, tomatoes, strawberries, melons, fresh onion, salads are fresh vegetable and fruit sources, employees in food industry can damage health in food production, food processing equipment and contaminated washing water together with contaminated drinking water, ice, not following hygiene and sanitation rules play important role in NoV infections (Atasever et al., 2015; Ray & Bhunia, 2016; Bintsis, 2017). Development of contamination in food takes place with transmission by infected employee that processes the product or cross contamination of products before arriving at restaurants and stores (employee hand, equipment and surface contacting food). Also NoV isolate analysis was conducted in mineral waters and NoV GIV was detected in waste water and rivers (Sökel & Kale, 2015). Food and water are sources of directly shaped contamination sources (especially food prepared with contaminated water) or there can be contamination during preparation. NoVs can stay persistently on environmental surfaces and food which is why it is difficult to prevent their infection. After comparison of Neurovirus strings collected from all around the world in the last ten years, researchers found that pandemic Neurovirus strains are carried with internationally sold food or travelers, increasing the possibility of tactile spread (Glass, 2009; Verhouf, 2011; Bintsis, 2017).

Some NoV symptoms in stomach-intestine infections are nausea, vomiting, fever, diarrhea (nonbloody), shivering, loss of water, and cramps. Usually children display vomiting while adults display diarrhea. Because of low fever and abdominal pain, this condition is also known as "Stomach Flu" (Chung, 2012). Symptoms are seen on average 24-48 hours following consumption of contaminated food. Incubation period is an average of 36 hours while findings of disease are seen in 72 hours. Contamination usually takes place in fecal oral route. NoV factors are mostly contaminating environment with feces and vomiting. Factor can usually stay in feces for 28 days (Erol, 2007; Atasever et al., 2015).

NoV cannot be replicated in organs and laboratory environment with classical cell cultures which is why Murine NoV, Feline Calicivirus (FCV) NoV, and Tulane Virus (TV) are the viral sources used in detection studies (Koopmans, 2002; Erol, 2007; Cook, 2016; Ray, 2016), various methods are used such as detection from patient human feces using electron microscope method, detection of antibody levels in blood against NoV or searching viral antigens with serological methods (Erol, 2007). Again, Lee et al. (2010), developed latex agglutination test (LAT) with tagged antibody latex bead method for detection of NoVs. Researchers demonstrated that LAT determines Nov GII types and that there was 35% and 100% harmony among methods in terms of sensitivity and originality using RT-PZR method (Lee et al., 2010). Park et al. (2008), combined RT-PZR and Immunomagnetic separation method (IMA) to ensure they complete each other and announced that they found successful results for food sourced NoV analysis. For NoV detection at ELISA, there must be 10^4 -10^5 virus particles (virions) in every gram of feces. At NoV diagnosis Nested PCR methods increasing PCR sensitivity and specificity are also commonly used (Sökel &Kale, 2015).

Rotaviruses

Rotaviruses are unenveloped, double stranded RNA viruses in *Reoviridae* family with 60-80 nm diameter (75 nm on average). Heat sensitive compared to other enteric viruses and is inactivated in 56 °C heat in 30 minutes. It is resistant to drying and disinfectants. It is resistant even between pH 3-10 (Erol, 2007; Atasever et al., 2015). Rotavirus is separated to 7 main groups (A to G) and 2 sub-groups. This variation depends on differences in Rotavirus inner capsid proteins (Atasever et al., 2015). However, only A, B, and C are capable of causing a disease (Öksüztepe & Demir, 2016). In Rotavirus infections mostly Group A Rotavirus Serotype G2 is responsible (Vasıckova et al., 2005; Atasever et al., 2015).

Rotavirus infections usually take place in fecal oral route and aerosol infection in winter months increases number of infections. Factor can stay alive in external environment for days, in river water (4-20°C) for some weeks, in room temperature and feces for months, on hands for 4 hours (Vasıckova et al., 2005; Atasever et al., 2015). While it is stable at 37°C for up to 4 days, at 56°C it is quickly deactivated. It also loses its vitality under UV light and with disinfectants such as chlorine, hydrogen peroxide,andethanol (auzefkitap.istanbul.edu.tr). Rotaviral infection can develop by contaminating tap water with sewer, using sewage at vegetable and fruit plantation, consumption of meat of an infected animal, consumption of contaminated food (fruits and vegetables) that are usually eaten raw after washing with contaminated water, consumption of shellfish gathered from areas contaminated with fecal source and infection of food with infected or carrier employees (Vasıckova et al., 2005; Erol, 2007). Food that get contaminated after cooking can also be source of viral infections (Vasıckova et al., 2005). When Rotaviruses enter body through oral route they are not completely inhibited in acidic environment of stomach. Infecting intestinal villi, Rotaviruses reproduce in cytoplasm of enterocytes, damaging transport mechanism to shape the disease. Incubation period is 1-3 days. (Atasever et al., 2015; Jaykus & Abarca, 2018). Clinical manifestation is acute gastroenteritis and usually begins with sudden vomiting and 1 or 2-day long fever which is followed by watery diarrhea for a few days (Jaykus & Abarca, 2018). Serogroup B is the type mostly seen in adults. Group C is the cause of diarrhea seen at children at any age group in many countries (Öksüztepe & Demir, 2016).

The disease displays vomiting, fever, diarrhea, and respiratory system symptoms. Rotavirus infections are frequently seen in common living environments (for ex. daycare centers, kindergartens, nurseries, and hospitals). Due to very low level of minimal infection dose, the infection is commonly seen (Erol, 2007). While Rotavirus is mostly infected with contaminated water rather than food, it is held responsible from childhood diarrhea that is spread globally. It is announced that at child patients breast milk is protective in 0-6-month period while for 24-month- old and older children partial immunization shaped with viral infection might result in mild or asymptomatic disease however, the infection causes severe disease at those with suppressed immune system (Atasever et al., 2015).

Rotavirus antigen can be detected in feces using antigen sets and immunochromatographic method or latex method or semi-nested type specific multiplex PCR methods following transcriptase (Ghalyoun & Alçay, 2017).

Hepatitis E Virus

Hepatitis E virus (HEV), is in *Hepevirus* type of *Hepeviridae* family. It is an unenveloped, enteric virus with 27- 34 nm (32 nm on average) diameter and single strand RNA. The virus is resistant to ether, chloroform, and detergents (Kırdar, 2012; Bosch et al., 2016; İncili & Çalıcıoğlu, 2016). Contrary to other enteric viruses, it is inactivated with factors such as frosting, high salt concentration, proteolysis, heating, and solutionizing (Erol, 2007). HEV has one serotype and 4 genotypes (genotype I, II, III, IV). Its endemic type in developing Asian and African countries is Genotype I, in Mexico and Africa there is genotype II, there is genotype III in America, some European countries and Japan there is genotype III including types isolated from sporadic cases together with Genotype IV seen in mainly Asian countries in human and domestic swine types. HEV is a zoonotic virus and is a frequent source of infection in swine and a lesser source of infection in humans (İncili & Çalıcıoğlu, 2016). Hepatitis E virus is found mostly in water contaminated with feces thus causing epidemics in especially countries devoid of hygiene conditions (Atasever et al., 2015).

HEV infects mainly in fecal-oral route while it also infects humans through blood and blood products, in perinatal and vertical routes. Virus spreads by infection of the factor on food by contaminated or carrier employee and causes foodborne infections in humans. After consumption of contaminated water and/or food virus reaches intestines and progresses to the liver. It mainly develops in liver and gall bladder cells and the multiplying factor is seen in intestine, lymph nodes, colon and salivary glands (Öksüztepe & Demir, 2016).

Following entry of HEV into the body, incubation period is 3 to 8 weeks (on average 6 weeks). The disease is usually mild and asymptomatic. Usually in 2 weeks recovery takes place by itself and in cases when recovery does not take place it causes symptoms and findings such as fatigue, loss of appetite, fever, vomiting, stomach and arthralgia, sensitive liver, hepatomegaly, and jaundice (http://auzefkitap.istanbul.edu.tr/kitap/laborantveveterinersaglik_ao/gghijyeni.pdf). While rate of mortality is 0.5-3% in case bile ducts are congested and cholestatic hepatitis is shaped and no recovery is achieved at this stage, it might cause serious death. (Jaykus & Abarca, 2018). Mortality rate that is 1% in healthy people can reach 20% in pregnant women (Koopmans, 2002; Atasever et al., 2015). The first Hepatitis E epidemic was seen in New Delhi in 1955-1956 when tap water was contaminated with sewage (Öksüztepe & Demir, 2016). Shellfish gown and/or collected in contaminated water is one of the main sources of infection in Hepatitis A and E epidemics while contamination of raw vegetables and fruit takes place with using contaminated water

during growing, processing, distribution, and preparation of products (Alp & Kuleaşan, 2018; Smith, 2001).

Adenovirus

This is an icosahedral symmetry virus that is a member of *Mastadenovirus* type of *Adenoviridae* family with a diameter of 70-90 nm (on average 80 nm), unenveloped, double stranded DNA (Vasıckova et al., 2005; Atasever et al., 2015). It might stay asymptomatically persistent in secretory glands and tonsils of children for years. Contrary to many viral infections it can create a long-term immunity against reinfection with the same serotype (Atasever et al., 2015; Ghalyeun & Alçay, 2017). In addition to being factor of mostly respiratory system diseases, following conjunctivitis and Rotavirus and Noroviruses, adenoviruses cause the highest gastroenteritis (especially in children age 4 and younger) (Ghalyeun & Alçay, 2017).

Adenoviruses were firstly isolated from human adenoid tissue in 1953 and for this reason was named adenovirus. In 1999 International Virus Taxonomy Committee separated adenoviruses to its types and serotypes. Among 9 human Adenovirus types, there are 7 types defined from A to G that frequently cause infection in humans (Alp & Kuleaşan, 2018). Adenovirus type 40 and 41 (Enteric Adenoviruses) are the biggest cause of diarrhea after Rotaviruses. Incubation period in gastroenteritis formation is 8-10 days, diarrhea can last for 5- 12 days and in some cases can pass 2 weeks. While symptoms such as fever, vomiting, cough, and diarrhea can be seen together with respiratory system infection findings can last for 1-3 days (Atasever et al., 2015). Adenoviruses can cause constipation due to mesenteric adenitis inflammation (Alp and Kuleaşan, 2018; Desselber & Gray, 2013).

While the main infection route of enteric adenoviruses is fecal-oral route, nonenteric adenoviruses are transferred with droplets and direct contact. It is notified that adenoviruses are detected in waste water, sea water, and shellfish. Waterborne adenovirus infections cause conjunctivitis in children while consumption of water contaminated with virus cause gastroenteritis form of the infection. Following entry into the body of the factor, the disease emerges in 3-10 days and diarrhea might last for 5-12 days (http://auzefkitap.istanbul.edu.tr/kitap/laborantveveterinersaglik_ao/gghijyeni.pdf). Infection can be seen anywhere but especially in gathering places of people such as hospitals, schools and nurseries (Öksüztepe & Demir, 2016). Mostly PCR methods are used in detection of adenovirus (Vasıckova et al., 2005).

Astrovirus

Identified in 1975, Astroviruses are in *Astroviridae* family and are 28-30 nm large, star-shaped with 5 or 6 corners. They take their name from the Latin word astron (star) (Atasever et al., 2015; Öksüztepe & Demir, 2016; Alp & Kuleaşan, 2018). They are among SRS viruses and might cause acute, viral gastroenteritis in children younger than age 1. Astroviruses have five different antigens and single stranded RNA. Astroviruses are stable at pH 3,0 and can live in 60°C. school age children have 64-87% antibodies against factor while this level was measured at around 80% in adults (Öksüztepe & Demir, 2016). Human astrovirus is the main cause of acute diarrhea in children and in some cases severity of the disease might necessitate treatment in hospital (Ghalyeun & Alçay, 2017).

The main factors in infection is fecal-oral route. Also, infection takes place through contaminated shellfish, tap water and food washed with water. Widely seen in winter season. The effect area of viruses are intestine mucosa epithelium and cause infection here leading to atrophy formation in villi. Among its clinical symptoms there can be mild diarrhea, abdominal pain, headache, fatigue and rarely vomiting. Critical frequency of can cause diseases with rotavirus and caliciviruses. Astrovirus infections are more common in winter months (Öksüztepe & Demir, 2016).

While astrovirus infections are directly related to food consumed raw, its epidemiology could not be described fully. The factor was seen in 50% of mussel samples and 17% of oyster samples (Vasıckova et al., 2005).

Electron microscopy, immunofluorescence of cellular culture and ELISA methods are used to diagnose the virus (Erol, 2007; Romero, 2011).

Coronavirus

Coronaviruses (CoVs) have zoonosis characteristic (cause disease in both humans and animals) and are enveloped, positive polarity viruses in Coronaviridae family with single stranded RNA (Ahn et al., 2020; http://auzefkitap.istanbul.edu.tr/kitap/laborantveveterinersaglik_ao/gghijyeni.pdf) According to their genotypic and serological structural properties they can be examined in four sub- groups, namely Alfa-, Beta-, Gamma- vet Delta coronaviruses (Marty & Jones 2020). Coronaviruses are common factors in many animal species starting with camels and bats. Some have the ability to infect among animals and humans. 7 different Coronavirus strands are known to cause infections in humans (http://auzefkitap.istanbul.edu.tr/kitap/laborant veveterinersaglik_ao/gghijyeni.pdf).

Coronaviruses were first defined by a veterinary doctor Dr. Oskar Siegfried in 1931 as factor of Infectious Bronchitis (IBV) in chicken (Shahidi, 2020). By mid-1960's humans, birds, mammals, and some other animals were completely defined to be infected with Coronavirus. The main target of factor in disease formation is respiratory system together with epithelium cells in gastrointestinal system. Until this day, seven coronaviruses have been identified to infect humans. In addition to common human coronaviruses, Betacoronavirus HCoVOC43 and HCoV-HKU1, Alphacoronavirus HCoV-229E causes widespread cold and serious lower respiratory tract infections in babies and elderly while false croup and bronchiolitis that Alphacoronavirus HCoV-NL63 causes in children is found to be significant. There are new zoonotic coronaviruses that come up in time causing epidemic in humans such as SARS-CoV (2002, Betacoronavirus, Sarbecovirus subtype) and MERS-CoV (2012, Betacoronavirus, Merbecovirus subtype). SARS-CoV-2 is closely related to SARS-CoV has a similar genetic structure as Sarbecovirus which is a subtype of Betacoronavirus (WHO, 2020). MERS-CoV and SARS-CoV mostly cause serious respiratory system infections. Findings related to SARS and MERS Coronaviruses demonstrate that virus that infects humans is not foodborne. In addition to these coronavirus types a new Coronavirus (nCoV) strain came about in 2019. Named as New betacoronavirus as International Taxonomy Committee, 2019-nCoV virus pandemic started at a local sea food market at Wuhan city in China. Research revealed that two thirds of 41 cases where the disease was detected were related to Huanan sea food market where there were also live animals (Sağdıç et al., 2020; Chen, 2020).

On February 27th, 2020 American Food and Drug Administration (FDA) notified that there were no concrete information on contamination of COVID-19 through food or food packages but added that ensuring prerequisite conditions of good hygiene applications and good production applications such as frequent cleaning and disinfection of hands and surfaces in chain of basic procedures such as processing, conservation, presentation, and distribution of food, keeping raw meat and such raw material sources separate from other food, cooking food in correct heat and rapid cooling were important criteria to prevent all food contaminations (FDA, 2020). They also notified that Coronavirus was detected in ice-creams produced in a town near capital of China, Beijing and employees of Daqiaodao Food company near Tianjin city where ice-cream was produced were tested for Covid-19. It was noted that ice-cream where virus was detected were confiscated and the

29.000 boxes containing products were not sold yet while those that had been sold, were recalled (https://medicalxpress.com/news/2021-01-

chinese-city-coronavirus-ice-cream.html). Report prepared by the World Health Organization informed that there was no information on foodborne infection of SARS-CoV-2 virus and that when previous coronavirus pandemics such as SARS-CoV and MERS-CoV were examined, no infection through food consumption were found while there were doubts about existence of such viruses in raw animal food (WHO, 2020).

Diagnosis and Detection Methods

Cultural enrichment is not a popular method due to low levels of virus particles in contaminated food and inexistence of standardized methods based on rapid culture. For this reason, before application of procedures related to the standard that shall be used in detection of viruses, particles must be concentrated in food matrix with molecular amplification which is the preferred detection method and purified. Organic solvents are used to concentrate and purify many viruses, ensure absorption or elution of the virus in food matrix and to ensure pH and ionic conditions are suitable to analysis. This is followed with comparably low speed centrifuging which would recover the virus containing phase (residue or eluate) for further purification. In other steps of virus concentration and purification, various types of filtration (raw filtration and ultra-fine filtration) are used. After virus concentration is increased, the relevant method for detection of genetic material can be used (Jaykus & Abarca, 2018). With molecular biology based methods, most common foodborne viruses (Norovirus, Hepatitis A and others) can be detected in shellfish or in water. However, usually none exist for other food (Vasicova et al., 2005). In detection of viruses in food tissue culture and embryo methods are also used since they are obligatory cellular microorganisms. Initially viruses are multiplied in contaminated food. In detection of Hepatitis A virus (HAV) special cellular culture procedures and immunological methods are used. In detection of both HAV and Norovirus (NoV) Revers-Transcriptase Polymerase Chain Reaction (RT-PCR) based on nucleic acid reproduction principle is widely used. With this method detection is made by multiplying viral RNA of HAV and NoV viruses. With enzyme immunoassay (EIA) method where antibodies are used against viral capsid proteins, specification methods based on genome strings are also used (Erol, 2007; Romero, 2011; Ray & Bhunia, 2016).

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Chapter 12

BIOFILM FORMATION AND SURVIVAL MECHANISMS IN FUNGAL, VIRAL AND BACTERIAL BIOFILMS

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Introduction

Biofilms are type of living of microorganisms under harsh environmental conditions. Many bacteria, fungi and even virus protect themselves from various effects by forming biofilms. These structures have gained relevance especially nowadays, as the ongoing pandemic, COVID-19, have had a worldwide impact.

Coronavirus (COVID-19) first started as the unclarified pneumonia in China in 2019. After that, more hospitilizations were reported, and Chinese authorities identified the virus as soon as determined the exposure to food in a fish market. Since many cases has been reported from many countries around the world, WHO suggested that the name of the virus would be COVID-19 and announced as a pandemic (World Health Organization, 2020).

Corona means 'crown' or 'halo' in Latin and it is given to the virus because of its appearance under the electron microscope. Coronaviruses (CoV) are belong to Nidovirales; enveloped-positive stranded RNA viruses which were identified as human pathogens more than 60 years ago. It is an infectious disease defined as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is included in the beta coronavirus family such as SARS-CoV (2002, Betacoronavirus, Sarbecovirus subgenus) and MERS-CoV (2012, Betacoronavirus, Mergencovirus subgenus) coronaviruses (European Centre for Disease Prevention and Control, 2020). Symptoms of COVID-19 are commonly high fever, cough, fatigue, breathing difficulties, loss of smell and taste. Symptoms can be seen either a day later or two weeks after exposure to Covid-19 or none. Some patients have mild symptoms, and some of them have acute respiratory distress syndrome, multi-organ failure, septic shock, and blood clots. Also, long term effect to organs such as in the lungs and heart has been reported. People also experience long COVID phase in which patients survived from the acute phase of the disease but still having fatigue, joint pain, chest pain, headache, memory loss and other cognitive issues, depression, fever, breathlessness, muscle weakness and pain, inflammation of the heart muscle, lung function abnormalities, acute kidney injury, rash, hair loss, smell and taste problems, sleep issues, difficulty with concentration, memory problems, depression, anxiety, and changes in mood for months afterwards (Centers for Disease Control and Prevention, 2020).

Although COVID-19 is a novel coronavirus, several research has been done to explain how it spreads, risk factors and risk groups, transmission, infection, clinical characteristics, diagnostic testing and screening, immune system responses and immunity, and also vaccines and treatments. However, the mechanisms are to be considered more to find

the appropriate treatments especially for the patients who have chronic diseases since the novel virus is still life threatening. Together with this, understanding the mechanisms of forming biofilm and survival strategies would reveal the chance of developing cure on patients suffering from chronic infections as well as pandemic related complications.

Bacterial Biofilms

Biofilms are group of microorganisms in EPS (extracellular polymeric substance) that survive by expressing different genes and producing essential proteins to adapt to harsh conditions (Gandhi& Chikindas, 2007; Cloete, Molobela, Van Der Merwe & Richards, 2009). Biofilm formation is a task beginning with an adherence of bacteria to the surface. Microorganisms require a substratum to attach and form mature biofilms. After the initial attachment, countable cells stay on the surface and remain attached irreversibly. Attached cells start producing extracellular protein (EPS). Finally, the cells grow on the surface and become mature biofilm (Figure 1). Planktonic bacteria have dissimilar properties comparing to biofilm growing bacteria. Being protected in a biofilm EPS matrix protects the cells, provides structural firmness and ensures bacteria to be closer to each other and increase virulence. In this case, diagnostic and therapeutic consequences become more relevant. Bacterial biofilms can contribute to food poisoning, microbially induced corrosion, reduce heat transfer efficiency, damage equipment and pipelines, accelerate corrosion and system clogging, pose hygienic risks in the food processing systems, dental or catheter biofilms (Donlan, 2001), and cause antibiotic resistance (Hoiby et al., 2010).

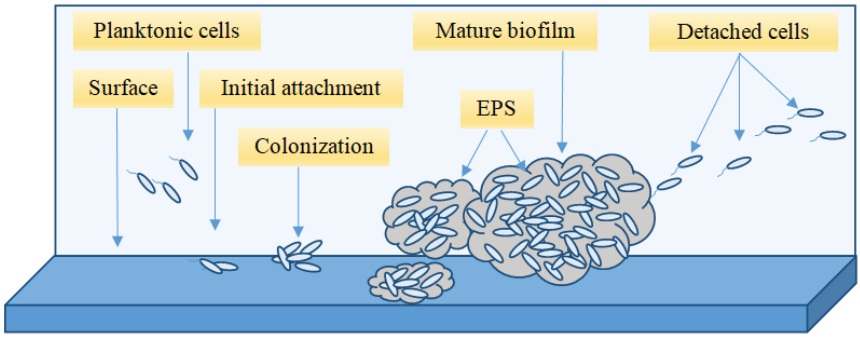


Figure 1. Biofilm formation steps: initial attachment, colonization, EPS production and biofilm growth, mature biofilm, detachment.

Biofilms are also very important in many diseases such as atopic dermatitis, psoriasis, leprosy (Allen et al., 2014; Allen et al., 2018; Allen &

Moschella, 2017), arthritis, otitis media, arteriosclerosis and Alzheimer's disease (Jacovides et al., 2012; Bakaletz, 2007; Allen et al., 2016; Moreau-Marquis, Stanton & O'Toole, 2008; Allen, Allawh & Goyal, 2018), gouty tophi and rheumatoid nodules (Allen, Cusack & Allen, 2019; Allen, Yi, Roberts & Allen, 2019), periodontit, and cystic fibrosis (Donlan & Costerton, 2002). Bacteria commonly cause this kind of infections either formed by a single species or multiple species.

Biofilm structure and properties varies depending on the surface properties, type of materials used, its contact time interval with the bacteria and ability to attach to surfaces or tissues. Moreover, the rate of biofilm formation depends on the count of attached cells and their types in the environment and on the substratum or devices. The flow rate of fluid through the surface, and the physicochemical properties of the substratum also affects the biofilm structure development. Other relevant factor affecting rate of attachment is surface properties which may be affected by the components in the surrounding liquid. Irreversibly attached cells start to produce extracellular polysaccharides to form a biofilm. Together with this, biofilm growth is affected by flow rate, nutrient composition of the surrounding liquid, antimicrobial concentration, and ambient temperature.

The gram-positive *Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*, and *Streptococcus* viridans*;* and the gram-negative *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis,* and *Pseudomonas aeruginosa* could mostly be isolated from medical devices. The skin of patients or healthcare workers and tap water that could be found in ports, or other factors in the environment could be the source of these bacteria (Donlan, 2001).

Antimicrobial Resistance

Once the biofilm is formed antibiotic resistance occurs. One or more of the following factors can be the reasons. One of them is slow or incomplete penetration of antimicrobial drugs into the biofilm (Stewart, 1996), development of a resistant phenotype (Fujita, 1992), adaptive responses (Stewart & Costerton, 2001), quorum sensing (Hoiby et al., 2010), genetic transfer, expression of resistance genes (Brooun et al., 2000), and persisters (Lewis, 2007). The extracellular matrix which can affect the penetration of antibiotics either by slowing their diffusion or by reacting with them, plays a relevant role. The EPS deposition increases as the biofilm matures and it causes a change in chemical gradients through the biofilm. Thus, oxygen distribution near the surface of the biofilms develops anaerobic conditions inside of the biofilm (Walters et al., 2003). Higher metabolic activity occurs with the oxygen concentration gradient on top of the biofilm and cells with low metabolic activity on the bottom

(Harmsen et al., 2010). This causes reduction in the growth rate of biofilm cells that results in differentiation into non dividing cells (Stewart, 1996). In *Pseudomonas aeruginosa* biofilms, the antibiotics interfering with processes such as replication (ciprofloxacin) and translation (tobramycin, gentamicin) specifically kill the cells on the surface of the biofilm without affecting the bottom layers (Harmsen et al., 2010). Bacteria in a biofilm can produce an autoinduction signaling molecule that accumulates in the environment when bacterial concentrations reach a critical number. Bacteria sense this high cell density and activate genes to produce virulence factors like enzymes and toxins which function as protective against antimicrobial agents (Fux et al., 2005; Hoiby et al., 2010, Simoes et al., 2010). In addition, quorum sensing plays a major role in bacterial activities like production of exopolysaccharide, development of biofilm, and virulence (Simoes et al., 2010).

Bacteria in biofilms can exhibit stress responses when encountering environmental challenges such as temperature change, DNA damage, oxidative stress and bacteria can undergo gene mutation when exposed to antibiotics (Stewart, 2002). The mutations in *P. aeruginosa* are reported to be in the methyl-directed mismatch repair genes that are required for DNA repair and DNA oxidative lesions repair. The oxidative stress which is related to production of reactive oxygen species leads to hypermutation in biofilms (Hoiby et al., 2010). Moreover, antibiotic resistance can be acquired through horizontal gene transfer between bacteria via conjugation. Genetic transfer in biofilms is higher than that in planktonic cells due to close contact of bacteria in biofilms (Foley & Gilbert, 1996). Expression of genes that contribute to antibiotic resistance is one of the defense mechanisms in biofilms. Production of β lactamases, aminoglycoside inactivating enzymes, and overexpression of multidrug efflux pumps are some additional mechanisms responsible for antibiotic resistance (Haddadin et al., 2010, Hoiby et al., 2010). β lactamases are enzymes inactivating β lactam antibiotics and are expressed by overproduction of gene AmpC (Hoiby et al., 2010). Similarly, overexpression of multidrug efflux pumps causes greater efflux of antibiotic out of the membrane, thus hindering the intracellular action of antibiotic.

Some cells in a biofilm have resistantance to antimicrobial therapeutics and develop as slow growing cells called persisters (Keren et al., 2004; Lewis, 2007; Mulcahy et al., 2010). When the antibiotic concentration decreases, they resume the growth and repopulate the biofilm. These persisters are also protected from host defences by the EPS (Lewis, 2007).

Either chemical or biochemicals which inhibit or destroy microorganisms are commonly used in biofilm control such as antibiotics which are used against infections in humans and animals. On the other

hand, antimicrobials called biocides are used in disinfecting objects and surfaces. The chemical methods used to control biofilms involve chemical agents, which can kill living organisms. Disinfectants such as hydrogen peroxide, quaternary ammonium compounds, and chlorine could be examples for these treatments (Van Houdt & Michiels, 2010). Biocides are effective on planktonic cells; however, when biofilms form, biocides may not be as effective in destroying bacteria (Simoes et al., 2010; Van Houdt & Michiels, 2010), possibly due to the EPS matrix (Van Houdt & Michiels, 2010) including diffusion barriers, differential metabolic activity, and cell-wall ultra structures (Mittelman, 1998). It was shown by Stewart et al. (Stewart et al., 2001) that *P. aeruginosa* PAO1 and *K. pneumonia* KP1 biofilm microorganisms had higher resistance to disinfectants when compared to their planktonic cells. In their study, alkaline hypochlorite or chlorosulfamate at a concentration of 1000 mg/l was applied both to biofilm and to planktonic bacteria; the planktonic bacteria were easily killed, but biolfilm bacteria were only partially killed. However, they also suggested that poor antimicrobial penetration was an insufficient explanation for biofilm resistance, because, although they found that the antimicrobial agent they employed effectively penetrated the biofilm, the results for biofilm killing were still poor. This suggests that bacterial cells in the film matrix were not only covered physically but also protected by other mechanisms. Frank et al. (1990) stated that, after the combined application of sodium hypochlorite and heat, mean reduction values in viable counts were about 100 times lower for *L. monocytogenes* biofilms than for planktonic cells. In another study (Folsom and Frank, 2006), thirteen strains of *L. monocytogenes* biofilms grown on stainless steel coupons were treated by 60 ppm chlorine solution. Eleven of these biofilms survived after being exposed to the chlorine solution. Robbins et al. (Robbins et al., 2005) studied the efficacy of ozone, chlorine and hydrogen peroxide on *L. monocytogenes* Scott A strain in both planktonic and biofilm form. They found that a 16-fold increase in sanitizer concentration was required to eradicate cells in the biofilm compared to planktonic cells. Depending on the type of biocide used, the effectiveness of the biocide to remove biofilms can vary. In one study (Vaid, Linton & Morgan, 2010), four day-old biofilms were formed from a mixture of five *L. monocytogenes* strains (Scott A, N1-227, 103M, 82, and 311) on stainless steel coupons. The effect of chlorine dioxide (CD) gas, aqueous CD and aqueous sodium hypochlorite on these biofilms were investigated. It was found that 0.3 mg/l CD gas, 7 mg/l aqueous CD and 50 mg/l sodium hypochlorite treatment of 10 min resulted in 3.21, 3.74, 3.09 log10 CFU/cm^2 reductions, respectively, from 4.80, 5.09, 4.95 log10 CFU/cm^2 of initial populations of biofilms respectively.

Moreover, when a multi-culture biofilm is developed, the resistance to biocide can be increased. Norwood et al. (2000) studied the effect of sodium hypochlorite on 28 day old biofilms of *L. monocytogenes* when grown in a multi-culture with *P. fragi* and *S. xylosus*. They obtained a 2-log decrease in bacterial numbers after a 1000 ppm free chlorine treatment, whereas 10 ppm of the same treatment was able to eliminate all the bacteria.

Viral Biofilms

Biofilms are commonly composed of the microorganisms, however, viral accumulation in viruses may occur that could help the virus to spread and make people sick and cause people suffer in chronic infections (Thoulouze & Alcover, 2011). The structural film of virus groups is supported by the infected host-cells. The type of viral transmission mechanisms is depends on the formation of extracellular viral particles whose structural properties and functions are similar to bacterial biofilms (Pais-Correia et al., 2000). The viral biofilms use the cell's DNA to replicate and compose the necessary amount of cells to form the biofilm.

Together with virus' capability from it´s capsule for survival and adherence to metal, plastic, surfaces, leads the way for future studies to determine if the virus is capable of surviving inside and in between the metal implants by forming viral biofilms.

Coronavirus (COVID-19)

Coronaviruses are large, enveloped, single-stranded positive-stranded RNA viruses belonging to the Coronaviridae family which have the largest genome amidst RNA viruses and having spike protein, the large surface protrusions that give the appearance of a crown; ensures virus to enter into the host-cells (Dhiman, Rakheja & Saxena, 2020).

The virus named COVID-19 is also known as SARS-Cov-2 or HCoV-19 which is a Beta-Corona which is RNA encapsulated. This capsule is formed by glucopolisacarides and a lipid layer which gives the virus it´s capability to adhere to cells and attach to them transmiting their RNA into the cell for viral proliferation.

In a study by Allen (2020), drugs use of hydroxychloroquine (HCQ) and azithromycin (Z) explained as they obtained beneficial effect in COVID 19 infection. It is suggested that the immune system causing tissue damage is activated by the biofilms created by virus. It is also stated that biofilms interacts with the immune system leading to disease as in psoriasis (Allen, 2020).

It is reported that biofilm formation on endotracheal tube became more relevant since extended intubation time span of COVID-19 patients and many developed superimposed pneumonias in the hospital because of ventilator-associated pneumonia (Sakano et al., 2020). In another research, mouth rinses found on the market have ingredients that could support to lower the SARS-CoV-2 viral load. Thus, the study suggests that the rinse facilitates the fight against oral transmission, and βCD-Citrox agent in oral biofilm rinses is successful in inhibiting the infection and controlling disease development (Carrouel et al., 2020).

In another study about the transmission and treatment of the virus, it is suggested that Covid-19 may also survive in sewerage. So, the researchers suggested that the use of nanofiber filters could have positive impact on wastewater treatment and upgrading of treatment systems could be more efficient (Venugopal, Ganesan & Sudalaimuthu Raja, 2020). Together with the inhibition of transmissions, developing novel treatments plays an important role. Wojewodzig (2020) stated that bacteriophages therapy could be potentially a suggested treatment (Wojewodzig, 2020) as depending on the concentration of viral units in each layer of biofilms mutant viruses could be protected from the immune system (Besharati et al., 2020).

Fungal Biofilms

Biofilm formation in fungi is relatively a new subject among biofilm researches comparing to bacterial biofilms. Fungal biofilms' development seems similar to those bacterial biofilms. For example, yeast cells must initially attach to the surface in the oral cavity, tissue or host cells in the environment before colonizing, interacting with other microorganisms and causing infections.

Most of the health related relevant fungi form film structures such as *Candida, Aspergillus, Cryptococcus, Trichosporon, Coccidioides, and Pneumocystis*.

Fungi cells in a film matrix are more resistant to antimicrobials such as antifungal drugs than planktonic cells. Complex structure, composition of extracellular matrix, metabolic heterogeneity, and biofilm-associated up-regulation of efflux pump genes are factors affecting resistance (Figure 2 & 3).

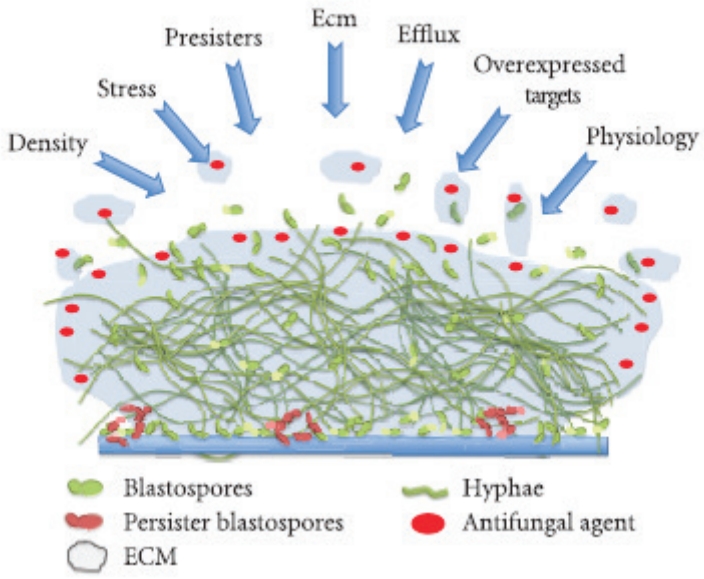
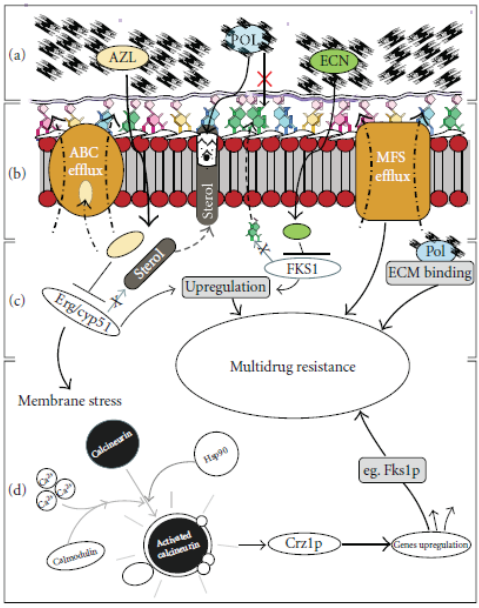


Figure 2: Schematic overview of fungal biofilm resistance mechanisms. Generic overview of key biofilm resistance mechanisms associated with *C. albicans*, but which are likely to be common to other fungi. This figure illustrates the density and complexity of the *C. albicans* biofilm, with different morphotypes present surrounded by ECM. The arrows represent the different factors that drive antifungal resistance within the biofilm, including density, stress, persisters, ECM, efflux, overexpressed targets, and the general physiology of the biofilm. These have been placed according to their contribution to resistance, with those that have a greater effect closer to the middle and those with less impact at the edges (Ramage et al., 2012).

Type of antifungal used, and biofilm species are relevant in resistance. *Candida albicans* and *Candida parapsilosis* biofilms are moderately unsusceptible to agents such as tofluconazole, amphotericin B, nystatin and voriconazole. When *Aspergillus fumigatus* forms biofilm, they become more resistant to itraconazole and, to caspofungin. *Cryptococcal* biofilm-structure showed resistance to fluconazole and voriconazole. *Trichosporon asahii* biofilms demonstrate high resistance to amphotericin B, caspofungin, voriconazole, and fluconazole. Azole and amphotericin B therapies are found unsusceptible on *Pneumocystis carinii* biofilms. Also, resistance mechanisms have been characterized in *C. albicans* and *A. fumigatus* biofilms including drug binding by EPS and existance of persister cells (Fanning & Mitchell, 2012).

Candida biofilms have many properties similar to bacterial biofilms which cause infections that are frequently resistant to antimicrobials. As a result of biofilm growth, cells are protected from host defenses (Jabra-Rizk, Falkler & Meiller, 2004).



Induced resistance gene		
Gene	Function	Resistance
Erg 1, 3, 11, and 25	Ergosterol	Cross-resistance
Kre1 Skn1	β-1,6 glucan	Polyene
Fks1	β-1,3 glucan	Cross-resistance
Zap1, Gcal1, 2, Adh5, Csh1	ECM regulators	Cross-resistance
CDR1–4	ABC transporters	Cross-resistance
Afu MDR4	ABC transporters	Azole
MDR1	MFS transporters	Azole
Mck1	MAPK	Cross-resistance
Hsp90	Heat shock protein 90	Cross-resistance

Extracellular matrix
β-glucan
Chitin
AZL Azoles
POL Polyenes
ECN Echinocandins

Figure 3: Molecular mechanisms of fungal biofilm resistance. Antifungal drug resistance in fungal biofilms is both complex and multifactorial. The diagram illustrates the mechanisms of different class of antifungal agent action (azoles [AZL], polyenes [POL], and echinocandins [ECN]) and resistance: (a) the layer of ECM present in the biofilm shields the cells from antifungal agents by binding and reduced penetration; (b) the membrane transporter system ABC and MFS efflux pumps extrude antifungal molecules and reduce the intracellular concentration; (c) mutation in ERG, Cyp51, and FKS1 genes alters the drug target leading to cross-resistance; (d) antifungal pressure induces stress responses, such as the calcineurin signalling pathway, which is activated, and coping responses occur through upregulation of various signal transducers. On the right hand side, the table lists different resistance genes and their functions, and antifungal agents affected (Ramage et al., 2012).

Conclusion

Biofilm formation and survival mechanisms in fungal, viral, and bacterial biofilms have unique mechanisms of biofilm formation and

survival strategies. Either fungal, viral or bacterial, the development of biofilms may lead to important medical situations in which pathogens may also include the fungi, bacteria or virus in the biofilm at the same time which may complicate the virulence. The importance of biofilms is considered vital especially in chronic diseases as well as hospitalization during pandemic. Revealing the biofilm formation and survival mechanisms would help people to find appropriate antimicrobials and methods to eradicate biofilms, and thus, the cells. However, more research should be carried out to understand these mechanisms better and develop strategies to fight biofilm forming cells effectively.

Acknowledgements

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Chapter 13

HISTOPATHOLOGY OF PEDIATRIC LIVER TUMORS

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Introduction

Hepatic masses constitute about 5-6% of all intra-abdominal masses in children. The majority of liver tumors in children are malignant; these malignant liver tumors constitute the third most common intra-abdominal malignancy in the pediatric age group after Wilms' tumor (WT) and neuroblastoma. Only about one third of the liver tumors are benign. A differential diagnosis of liver tumors in children can be obtained based on the age of the child, clinical information (in particular AFP) and imaging characteristics (Jha et al, 2009; Ng and Mogul, 2018).

Liver tumors in childhood are rare and are typically not detected clinically until they reach a large size and often spread within the organ or metastasize. This can make surgical resection problematic, and almost all of them require extirpation for cure. With very effective chemotherapy for hepatoblastoma (HB) and to some extent for sarcomas, many cancers can be shrunk to permit partial hepatectomy, but for most hepatocellular carcinomas (HCC), some of the other malignancies, and even some benign proliferations, their location at the hilum and multiplicity of masses in multiple lobes make transplantation the treatment of choice. Major advances in diagnostic imaging, especially enhanced computed tomography (CT) and magnetic resonance imaging (MRI), permit a preoperative choice of resection versus transplantation to be achieved in almost all instances, and for the remainder, intraoperative ultrasonography (US) can further help to determine the most desirable approach. The outcome is very much better in the case of HB when transplantation is a primary modality rather than following unsuccessful attempts at resection (Finegold et al, 2018).

Although rare, hepatoblastoma (HB) is the most frequent of the malignant pediatric liver tumors. After neuroblastoma and nephroblastoma, HB is the third most common abdominal solid tumor in very young children. Hepatocellular carcinoma (HCC) is most often seen in older children and adolescents. An interesting hybrid tumor, HB with features of HCC variably called transitional or "not-otherwise-specified" (NOS), exists on the age continuum between HB and HCC. In Asia and Africa, HCC occurs more frequently than HB, which is probably a consequence of the higher prevalence of hepatitis B infection on those continents. The incidence of HCC seems to have decreased in some countries as a consequence of wide spread vaccination programs against hepatitis B infection in these areas (Moore et al, 2008). In today's age of electronic communication, the possibility of collaboration on an international scale has increasingly facilitated treatment and research of these rare tumors (Aronson and Meyers, 2016).

The purpose of this review is to provide both clinicopathologic and histopathologic guide for the pediatric liver tumors, especially for the malignant childhood neoplasia.

Table 1. *2014 - International Consensus classification of the pediatric liver tumors* **(Lopez- Terrada, 2014).**

Epithelial tumors	Mesenchymal tumors
Hepatocellular[a]	Benign
Benign and tumor-like conditions	Vascular tumors
Hepatocellular adenoma (adenomatosis)	Infantile hemangioma
Focal nodular hyperplasia	Mesenchymal hamartoma
Macroregenerative Nodule	Pecomas
Premalignant lesions	Malignant
Dysplastic nodules	Embryonal sarcoma
Malignant	Rhabdomyosarcoma
Hepatoblastoma	Vascular tumors
Epithelial variants	Epithelioid hemangioendothelioma
Pure fetal with low mitotic activity	Angiosarcoma
Fetal, mitotically active	*Other malignancies*
Pleomorphic, poorly differentiated	Tumors of uncertain origin
Embryonal	Malignant rhabdoid tumor
Small-cell undifferentiated	**INI1− (documented *INI1* mut)**
INI1-negative	INI1+
INI1-positive	Nested epithelial stromal tumor
Epithelial mixed (any/all above)	Other
Cholangioblastic	***Germ cell tumors***
Epithelial macrotrabecular pattern	Teratoma
Mixed epithelial and mesenchymal	Yolk sac tumor
Without teratoid features	DSRCT
With teratoid features	PPNET

Hepatocellular carcinoma		***Metastatic (and secondary)***
Classical HCC		Solid tumors (NB, WT, other)
Fibrolamellar HCC		Acute myeloid leukemia (M7)
Hepatocellular neoplasm NOS[b]		
Biliary		
Benign		
Bile duct adenoma / hamartoma, other		
Malignant		
Cholangiocarcinoma		
Combined (hepatocellular cholangioca)		

Abreviations: DSRCT: desmoplastic small round cell tumor, PPNET: peripheral primitive neuroectodermal tumor, NB: neuroblastoma, WT: Wilms'Tumor Note: associated genetic syndromes, malformations, familial cancer syndromes, metabolic disorders, prematurity, and underlying liver disease should always be reported when available. a Classification applies only to pre-chemotherapy specimens. bIndicates provisional entity. bTumors previously designated as transitional liver cell tumors may be included in this category.

Children's Hepatic International Collaboration (CHIC), Children's Oncology Group (COG), Société Internationale d'Oncologie Pédiatrique (SIOP), and International Childhood Liver Tumors Strategy Group trials have contributed to define prognostic factors and risk stratification in these tumors. The histopatologic International Consensus classification of HB and HCC in children based on retrospective analysis from CHIC cases represents the base to define entities with homogeneous clinicopathologic and molecular features (Ranganathan et al, 2020).

Hepatoblastoma (HB)

The treatment of HBs has been one of the success stories of pediatric oncology. From a disease with a dreadful outlook with survival rates around 35% in 1970s, survival has now reached over 90% for patients with standard risk tumors, and 45–80% for patients with metastatic disease (Zsiros et al, 2013). Progress has been made due to improved surgical technique and better risk stratified chemotherapy based on increasingly refined risk factors. Identifying poor molecular prognostic factors such as the NFR2 mutation and a high risk 12 gene signature may open the way to the development of new personalized targeted therapies in the future (López-Terrada et al, 2014).

Clinical Features of HB

Hepatoblastoma comprises 1% of all pediatric malignancies. It occurs equally in males and females, most often presenting in infants and young children between 6 months and 4 years of age, with a median age of onset of 18 months. It is rare after 5 years of age and seems to behave more aggressively in children over 8 years (Czauderna et al, 2016). Cases in neonates and adolescents have also been reported.

Pateva et al performed search for HB and epidemiology reports occurring in children between the ages of 5 and 18 years and review of SEER program data shows that the incidence of HB in children above the age of 5 years is too infrequent to be calculated. Literature review revealed 13 cases of patients diagnosed at age older than 5 years (Pateva et al, 2017). HB most commonly disseminates to the lungs and very rarely to the local abdominal lymph nodes, neither of which are associated with symptoms or clinical signs. Most cases are sporadic but some are associated with genetic cancer syndromes like Beckwith–Wiedemann syndrome, Familial Adenomatous Polyposis or trisomy 18/Edwards syndrome and suggest a role in the pathogenesis of HB for chromosomes 5, 11, and 18, respectively (Czauderna et al, 2014). Also prematurity and very low birth weight have been associated with HB, and increases in premature birth rates have been postulated to drive an increase in HB incidence (Spector and Birch, 2012).

Measurement of AFP concentrations in the serum of infants is useful for the management of HB. Preterm and term infants up to the 3rd month of life exhibit the highest average AFP concentrations (Ferraro et al, 2019).

Histopathologic Subclassification of HB

HB has two types, the wholly epithelial type and the mixed epithelial and mesenchymal (MEM) type. The wholly epithelial type was subdivided into well-differentiated fetal (pure fetal with low mitotic activity), crowded fetal (mitotically active), embryonal, epithelial mixed, small cell undifferentiated, and cholangioblastic. A macrotrabecular pattern and a pleomorphic epithelial pattern were recognized as supplemental features of epithelial components. The MEM type was subdivided into MEM without teratoid features and MEM with teratoid features. Other liver cancers in children were divided into hepatocellular carcinoma (classic HCC and fibrolamellar HCC) and hepatocellular malignant tumor not otherwise specified (Table 2). This classification is basically applied to pretreatment specimens; the evaluation of post-chemotherapy specimens will be the subject of further studies (Tanaka et al, 2013). HB is an embryonal tumor believed to arise from hepatic progenitor cells that are arrested at various stages of liver development, as illustrated

by a variety of histologic subtypes. These tumors are rarely composed of only one cell type, but usually very heterogeneous with combinations of epithelial, mesenchymal, undifferentiated, and other histologic components. The rarity of these tumors, even in specialized institutions, makes their diagnoses challenging for the general pathologist, and consultation with an experienced pathologist is encouraged. For most tumors, histopathologic classification does not determine prognosis. So far clinical trials have demonstrated that only two histological types seem to be associated with prognosis: well differentiated fetal (WDF) and small cell undifferentiated (SCU) (Meyers et al, 2009). Several studies have demonstrated that WDF histology, accounting for about 7% of all HB and composed only of cells resembling fetal hepatocytes with minimal mitotic activity, correlated with improved survival. Part of the definition is that the tumor has not been treated with chemotherapy and is completely resected. In a Children's Oncology Group (COG) publication, Malogolowkin et al. reported on 25 patients with WDF histology and low mitotic activity, staged PRETEXT I-III who were treated with surgery only. These patients showed 100% event-free survival (EFS) (Malogolowkin et al, 2011). It is important to remember, though, that most HB are extremely heterogeneous, often with closely intermixed histological components, and only rarely composed of a single histological type.

A series of recent international collaborative pathology symposia have yielded a new pathology consensus classification shown in Table 2. The new global classification includes all prognostically relevant histological types (WDF and SCU), as well as the new categories ("pleomorphic epithelial" and "malignant hepatocellular neoplasm"), recognizing how challenging some tumors are to classify, particularly after chemotherapy, recommendations were outlined for submission, sampling, and evaluation of pediatric liver tumor samples, including minimum diagnostic specimen requirements and evaluation of the uninvolved liver, as well as the necessity of providing minimal clinical information to the reviewer, which should always include age, AFP level at the time of diagnosis, underlying liver disease, and correlation with imaging (López-Terrada et al, 2014). There was also consensus between the pathologists regarding the importance of obtaining pre-chemotherapy specimens for the initial diagnoses and tumor classification. Finally, the group also pointed to the importance of tissue banking for biological studies. Specific caution needs to be taken when assessing tumors in older children in which Hepatocellular Neoplasms-not otherwise specified (HCN-NOS) with dual features of HB and HCC, and also HCC (Buendia, 2014).

A second histological subtype of interest is the small cell undifferentiated HB (SCU-HB). This histologic pattern may be associated

with low serum AFP levels and poor response to chemotherapy. The first report regarding the negative association of SCU component was by Kasai and Watanabe (Kasai and Watanabe, 1970); followed by Haas's Children's Cancer Group–Pediatric Oncology Group (CCG–POG, also referred to as COG legacy groups) report, in which none of the patients with SCU-HB survived 24 months after the diagnosis. Trobaugh-Lotrario et al. reviewed 11 patients with SCU-HB presenting with clinically normal or minimally increased serum AFP levels, none of whom survived (Trobaugh-Lotrario et al, 2009). There has been an increased emphasis on the need to distinguish SCU-HB from malignant rhabdoid tumors of the liver which are INI-1 negative (discohesive, eccentric irregular nuclei, prominent nucleoli, abundant cytoplasmic filaments including cytokeratin and vimentin, negative nuclear INI).

Table 2. *2014 - International consensus classification of the histopathological subtypes of hepatoblastoma (Aronson & Meyers, 2016; Lopez-Terrada et al, 2014).*

Epithelial	**Subtype / criteria**	**Mixed**	**Subtype / criteria**
Fetal	**Well differentiated** and uniform (10–20-µm diameter), round nuclei, cords with minimal mitotic activity (<2 per 10 HPF), EMH	Stromal derivatives	**Spindle** cells (blastema), osteoid, skeletal muscle, cartilage
	Crowded or mitotically active (>2 per 10 HPF); conspicuous nucleoli (usually less glycogen)	Teratoid	**Mixed**, plus primitive endoderm; neural derivatives, melanin, squamous and glandular elements
	Pleomorphic, **poorly differentiated** moderate anisonucleosis, high N/C ratio, nucleoli		
	Anaplastic with marked nuclear enlargement and pleomorphism, hyperchromasia, abnormal mitoses		
Embryonal	10–15- µm diameter, high N/C, angular, primitive tubules, EMH		
Macrotrabecular	Epithelial HB (fetal or embryonal) growing in clusters of >5 cells between sinusoids		

Epithelial	Subtype / criteria	Mixed	Subtype / criteria
Small cell undifferentiated (SCU)	5–10- μm diameter, no architectural pattern, minimal pale amphophilic cytoplasm, round to oval nuclei with fine chromatin and inconspicuous nucleoli, ± mitoses; ± INI[a]		
Cholangioblastic	Bile ducts, usually at periphery of epithelial islands, can predominate		

[a]*EMH, extramedullary hematopoiesis.* [b]*Pure small-cell undifferentiated needs to be differentiated from malignant rhabdoid tumors (discohesive, eccentric irregular nuclei, prominent nucleoli, abundant cytoplasmic filaments including cytokeratin and vimentin, negative nuclear INI).*

The developing liver displays many features similar to those seen in HB, including uniform hepatocytes and cords two cells thick separated by sinusoids displaying hematopoiesis. HBs display only minimal ductular differentiation, similar to the fetal development of the liver that does not display significant ductular development until well into the second trimester (Stocker, 1994).

Radiographic Staging (including PRETEXT) and Risk Stratification of HB Staging is essential for risk categorization, risk adapted treatment and prognostication. The commonest staging and risk categorization system used today is PRETEXT (PRE-Treatment EXTent of tumor) system that is being used by nearly all multicentre trials (American, European, German, Japanese) in some way (Agarwala, 2012). Imaging is crucial in the assessment of children with a primary hepatic malignancy. Either contrast enhanced computed tomography (CT) or MR is recommended. Many radiologists report that MRI angiography enhanced by hepatocyte specific contrast agents such as eovist gadolinium may improve differential diagnosis and detection of small disease deposits that can be seen with multifocal disease (Meyers et al, 2012; Voss, 2018). Since its inception in 1992, the PRETEXT system has become the primary method of risk stratification for HB and pediatric HCC in numerous cooperative group trials across the world. The PRETEXT system is made of two components: the PRETEXT group and the annotation factors (Table 3). The PRETEXT group describes the extent of tumor within the liver while the annotation factors help to describe associated features such as vascular involvement (either portal vein or hepatic vein/inferior vena cava), extrahepatic disease, multifocality, tumor rupture and metastatic disease (to both the lungs and lymph nodes).

Table 3. *PRE-TEXT Prognostic factors and their classification – 2017 (Meyers et al, 2017)*

PRETEXT group	**I** => one section involved, three sections tumor free;
	II =>one or two sections involved, two sections tumor free;
	III => two or three sections involved, one section tumor free;
	IV =>four sections involved*
PRETEXT annotation factors	sections involved*
V: involvement of vena cava— all three hepatic veins or the intrahepatic inferior vena cava, or both	Yes *vs* No
P: involvement of portal vein— both left and right portal vein, or portal bifurcation, or both	Yes *vs* No
E: contiguous extrahepatic intra-abdominal tumor extension— contiguous involvement of adjacent organs (eg, diaphragm and bowel)	Yes *vs* No
F: multifocal liver tumor— two or more tumor nodules separated by normal hepatic parenchyma	Yes *vs* No
R: tumor rupture at diagnosis	Yes *vs* No
M: metastasis— non-contiguous tumor spread, usually to the lungs	Yes *vs* No
AFP concentration, ng/mL	≤100 *vs* 101–1000 *vs* 1001–1 000 000 *vs* ≥1 000 000
Age, years	0 to <1 *vs* 1 – 2 *vs* 3 – 7 *vs* ≥8

PRETEXT: PRETreatment
EXTent of disease,
AFP: α fetoprotein

It has been one of the earliest achievements of the SIOPEL group to produce a preoperative staging system (PRETEXT), based on radiological imaging. A non-surgical, radiographic system had to be developed when SIOPEL began treating all patients with primary chemotherapy in the 1980s. The North American approach at this time was surgical staging based on attempted resection at diagnosis. Over the years it has become clear that while small localized tumors may be amenable to up-front resection, patients with extensive tumors may benefit from preoperative chemotherapy and the PRETEXT staging groups (I, II, III, IV) which define extent of liver parenchyma involvement have stood the test of time. The PRETEXT annotation factors which help to define the extent and nature of any extrahepatic disease have evolved over time. Initially just V, P, E, and M, these factors now include V, P, E, F, R, N, C, and M defined as tumor involvement of the hepatic veins or retrohepatic vena cava (V); main portal or portal bifurcation (P); contiguous organ such as diaphragm, abdominal wall, bowel, etc. (E); multifocal tumor nodules (F); tumor

rupture at diagnosis (R); lymph nodes (N); caudate lobe (C); and distant metastatic (M). PRETEXT group is highly predictive of outcome and this system has been adopted as the common international language for HB staging now used in some form by all major multicenter trial groups (Roebuck et al, 2007).

Successful margin-negative resection of HB is dependent on the location and extent of disease as defined by the PRETEXT classification. Accurate preoperative staging is vital to the current clinical SIOPEL trials portfolio where low-risk tumors (PRETEXT I–III) are managed quite differently from high-risk tumors (PRETEXT IV and metastatic) (Aronson et al, 2014). The thrust for high-risk tumors remains driving unresectable tumors to become resectable whilst dealing with metastatic disease through an intensive chemotherapy schedule. COG currently uses the risk categories very low, low, intermediate, and high-risk to assign treatment strata. Assignment into these risk categories is based on a hybrid system which retains elements of the old surgical based Evan's staging system (stage 1 = resected at diagnosis; stage II= attempted resection at diagnosis with gross residual tumor; stage III = preoperative chemotherapy; and stage IV = metastatic at diagnosis) while defining the appropriate time for surgical resection based upon PRETEXT and POST-TEXT. Surgery at diagnosis is recommended for PRETEXT I or II tumors with preoperative radiographically clear venous margins. Primary chemotherapy and delayed surgery is recommended for PRETEXT III and/or POSTTEXT I, II, or III with no major venous involvement (V- and P-). Finally, both SIOPEL and COG recommend any PRETEXT IV and/or POSTTEXT IIIV+P+ to best be referred early to a liver specialty center with transplant capability. As complete resection is essential for cure and well predicted by PRETEXT, all major liver tumor study groups now use PRETEXT stage within their system of risk assessment (Table 3).

Diagnosis of the pediatric liver tumors can be challenging because of their rarity, and the recognition of distinctive imaging features for certain tumors such as epithelioid hemangioendothelioma and biliary rhabdomyosarcoma can focus the differential diagnosis and expedite the diagnostic process (Chavhan et al, 2019). The unique imaging features of hepatic neoplasms in the pediatric population, when coupled with supportive demographic data and laboratory findings, can lead to accurate diagnosis and proper treatment of hepatobiliary tumors (Yikilmaz et al, 2017).

Chemotherapy and Survival of HB

Throughout the past decades, the evolution of chemotherapeutic approaches have shown a decrease in toxicity for localized disease and an increased intensity for high-risk tumors. Results of randomized COG and

SIOPEL studies in patients with localized disease (standard-risk tumors) have shown that cisplatin is the best agent in the treatment of this subgroup of patients and it became clear that doxorubicin and its associated toxicities could be safely omitted from the treatment without endangering the long-term outcome (Perilongo et al, 2009). Very low and low-risk are PRETEXT I and II resected at diagnosis, and intermediate-risk are non-metastatic PRETEXT II–IV patients who received preoperative chemotherapy. The very-low-risk received no postoperative chemotherapy (Malogolowkin et al, 2011). All of these cisplatin based chemotherapy regimens carry a high risk of ototoxicity.

Orthotopic Liver Transplantation (OLT)

Surgical resection remains a prerequisite for cure. Resection rates have increased over the years through intensification of chemotherapy for high-risk tumors, and increased use of extreme resections and orthotopic liver transplantation (OLT). For unresectable tumors, total hepatectomy and OLT have been used increasingly after it had been shown that its survival rates equaled survival rates after complete/partial hepatectomy. In this mile stone article from 2004, Otte showed that a primary procedure ("primary" transplant) resulted in an 87% long time disease free survival. In contrast to these results, overall survival dropped to 30% in those in whom a "rescue" transplant was performed after a prior incomplete resection or a local recurrence following partial hepatectomy (Otte et al, 2004). Based on these results, the percentage of patients with POSTTEXT IV tumors who underwent a liver transplantation have markedly increased. The so called extreme resections as done by some experienced liver surgeons are a potential alternative to OLT in some patients, but are only advocated by experienced teams in the setting of a transplant safety net (Meyers et al, 2014). Even children who present with metastatic disease at diagnosis may eventually become illegible for OLT if metastatic remission with preoperative chemotherapy has been achieved. Otherwise, residual small pulmonary nodules that may either contain viable tumor cells or just scar tissue must then first be removed in order to have histopathological proof that the lung disease has been cleared (Otte et al 2004). The current guidelines for OLT are (1) either POSTTEXT IV or POSTTEXT III with major vascular involvement (V+, P+); or (2) extensive multifocal tumors that carry an increased risk of local relapse. Ideally the patient should also have demonstrated some response to chemotherapy (tumor reduction) and a serial fall of AFP (Meyers et al, 2012). As the liver transplantation should be carried out within a few weeks after the third or fourth course of chemotherapy, the option of a live-donor liver transplantation has the advantage that it can be planned for a specific time.

Controversy continues to surround the ideal approach to a multifocal (F+) PRETEXT IV tumor that downstages to a POST-TEXT III, F+ with radiographic, but not necessarily microscopic, tumor clearance of at least one section. The trial group guidelines of SIOPEL and COG remain in a safety posture advising OLT, although success has been achieved by some with aggressive resection in this setting (Czauderna et al, 2005).

Ototoxicity

A serious permanent side effect of cisplatin therapy is bilateral high-frequency hearing loss which is particularly debilitating when it occurs at a young age (Landier et al, 2014). The risk of ototoxicity is significantly increased in children younger than 5 years of age with higher cumulative doses of cisplatin received. However, because the predisposition to cisplatin hearing loss is individual, even low cumulative doses in some children can cause damage. The SIOPEL-6 study investigated sodium thiosulfate (STS) as an agent to decrease the cisplatin induced ototoxicity. STS has been shown to dramatically reduce hearing loss in children treated with cisplatin containing chemotherapy without tumor protection in localized disease.

Treatment and Prognosis

The management of HB has changed markedly over the last three decades; neoadjuvant chemotherapy is now standard, particularly in unresectable tumors resulting in considerable preoperative tumor shrinkage and sometimes near total ablation of the tumor (Sharma et al, 2017). Treatment of HB is multimodal with surgery and chemotherapy being the main modalities. Survival is not possible without complete surgical resection. Majority of tumors are unresectable at presentation but can be made resectable with chemotherapy, giving a resection rate of more than 85%. Cisplatin is the main stay of chemotherapy and is a part of all multidrug protocols. The 3-y overall survival (OS) today stands at 62-70% but only 25% patients with metastasis get cured. Panhepatic tumors and those with local factors causing unresectability are now dealt with liver transplantation which has also given a survival rate of nearly 85%. The overall management of HB and HCC has evolved over the past three decades giving good long term survival rates for HB, though patients with HCC still do poorly. Successive therapeutic trials have focused attention on increasing the efficiency and reducing the toxicity and long term side effects of the treatment (Agarwala, 2012).

Liver transplantation is an appropriate treatment modality when complete oncological resection requires total hepatectomy. In general, advanced PRETEXT class as well as histopathologic features, age at

presentation, tumoral production of α-feto protein and the presence of metastatic disease adversely affect outcome. HB is chemosensitive and significant downstaging can occur with the use of neoadjuvant chemotherapy allowing for less extensive hepatectomy. In addition, patients at moderate-to-high risk of postresection recurrence should receive neoadjuvant chemotherapy. Cisplatin-based chemotherapy can allow for resection of transplantation of patients with metastatic disease when complete metasatectomy can be achieved albeit with inferior results (Hafberg et al, 2019).

Transarterial chemoembolization is a minimally invasive locoregional treatment option performed by interventional radiologists with level-I evidence as standard of care in adults with advanced liver malignancy; transarterial chemoembolization in adults has served to prolong disease-free progression, downstage and bridge patients for surgical and transplant interventions, and improve overall survival. However, while several groups have reported that transarterial chemoembolization is feasible in children, the published experience is limited primarily to small retrospective case series. The lack of prospective trial evidence has in part limited the utilization of transarterial chemoembolization in the pediatric patient population (Lungren et al, 2018).

Hepatocellular Carcinoma (HCC)

Pediatric hepatocellular carcinoma (HCC) is the second common malignant liver tumor in children after HB. It differs from the adult HCC in the etiological predisposition, biological behavior and lower frequency of cirrhosis. Perinatally acquired hepatitis-B virus, hepatorenal tyrosinemia, progressive familial intrahepatic cholestasis, glycogen storage disease, Alagille's syndrome and congenital porto-systemic shunts are important predisposing factors. Majority of children (87%) are older than 5 years of age (Khanna and Verma, 2018).

The incidence of HCC in the western world seems stable or slightly increased (Kelly et al, 2015). In adults, HCC occurs most commonly in the setting of chronic cirrhotic liver disease. In children, however, there are several distinct clinical and biologic patterns of HCC which cluster into two broad categories. First are the sporadic or "de novo" tumors where HCC arises in a normal liver without preceeding liver disease. An ongoing debate questions whether this de novo form of pediatric HCC biologically differs from HCC in adult cirrhotic livers. These de novo tumors may exhibit variable histologies: transitional type tumors with features of both hepatoblastoma (HB) and HCC (also called HepatoCellular Neoplasm-not otherwise specified [HCN-NOS]) (López-Terrada et al, 2014), conventional HCC, and fibrolamellar HCC (FL-

HCC). The second broad category of pediatric HCC include tumors which develop in the context of chronic or congenital liver disease. HCCs of this latter subtype are most commonly described in patients with chronic viral hepatitis or one of a variety of congenital diseases including glycogen storage disease, cholestatic syndromes, such as Alagille's, metabolic and autoimmune syndromes including hereditary tyrosinemia, Wilson's disease, hemochromatosis, alpha-1 antitrypsin deficiency, autoimmune hepatitis, and primary sclerosing cholangitis. Rare reports have linked HCC to ataxia telangiectasia, familial cholestatic syndromes, transaldolase deficiency, Gardner's syndrome or familial adenomatous polyposis, and Fanconi anemia (Kelly et al, 2015). HCC developing in the context of congential liver disease may sometimes be diagnosed early secondary to vigilant screening.

Moore et reviewed pediatric liver tumors in South Africa and reported that 57% of the tumors were HB, 35% were HCC, 5% were sarcoma of the liver, and remaining were lymphoma. The ratio of HB to HCC (1.67) was markedly lower compared with other reports, suggesting a greater prevalence of HCC (Moore et al, 2008).

Physical signs and symptoms may include abdominal pain, gastric reflux, early satiety, abdominal mass, hepatomegaly, and in advanced cases, anorexia and weight loss. Rarely there may be acute decompensation from tumor rupture or GI bleeding (Ni et al, 2004). Although the literature reports a wide and disparate range, it seems that only about 55–65% of children with HCC present with elevated alpha-fetoprotein (AFP). But even when elevated, the AFP is generally not as high as in children with HB (Katzenstein et al, 2002). Patients with chronic liver cirrhosis may have low-level elevations in AFP at baseline; therefore levels of >100 ng/mL or higher, or an AFP that is trending upward over time, should prompt concern.

Ideal diagnostic imaging would be abdominal MRI with liver specific contrast agents and a chest CT for evaluation of pulmonary nodules. Spread of disease to the bone and brain is exceedingly rare. CT is less effective than MRI with gadoxetate-disodium (Eovist), in detecting small lesions particularly in the context of cirrhosis. MRI with gadoxetate-disodium (Eovist), a contrast agent with a prolonged hepatocyte parenchymal phase, is the most reliable way to detect the small lesions of multifocal tumors, which are seen in up to 50% of cases (Vasanawala and Lustig, 2011).

Historically various pediatric HCC analyses have used one of three different staging systems: Evan's Stage I–IV in America; PRETEXT Group I–IV with annotation factors (VPEM) for vascular involvement, extrahepatic, and distant metastasis in Europe; and the TNM system in

adults (Kelly et al, 2015). The PRETEXT annotation factors, originally described as VPEM for early studies of HB by SIOPEL, have been subsequently expanded to include multifocality (F), tumor rupture (R), caudate involvement (C), and lymph node involvement (N) (Meyers et al, 2014).

Because HCC is relatively chemoresistant, if at all possible any child without metastasis should be considered for surgical resection at diagnosis, even if the resection will be technically difficult or require liver transplant. The role of liver transplantation in pediatric HCC has recently been increasing.

In adult HCC, transplantation guidelines generally adhere to Milan criteria. However, it is the current thinking that the Milan criteria may not be applicable in children because they are derived from experience in adults with cirrhosis, which most children with HCC do not have (Murawski et al, 2016).

The role for post-operative chemotherapy in these resectable-at-diagnosis patients is not clear. Patients who had a resection up front seemed to have a better outcome than after neo-adjuvant chemotherapy and delayed surgery. Despite tumor response in 40–59% of patients, results remained dismal with overall survival rates of less than 30% (Murawski et al, 2016). So far, tumor resectability appears to be the most important factor for survival as lymphovascular invasion, extrahepatic tumor, and metastatic disease precluding complete resection, are poor prognostic factors (<10% 5-year EFS) (Czauderna et al, 2002).

The published experience regarding pediatric liver transplant for HCC is limited but a review of pediatric HCC in the SEER database, showed 5-year survival was 53.4% after conventional resection and 85.3% after transplant (McAteer et al, 2013). Neoadjuvant chemotherapy may be considered while awaiting receipt of an organ. Unfortunately most patients present with metastatic disease, and for metastatic disease the prognosis is poor with 5-year EFS only 15–20%.

Some have suggested pediatric HCC, on average, may be more chemosensitive than that seen in adults. Whether this is due to age alone, possible pediatric study inclusion of transitional type cases of HB with HCC features, or perhaps a biologic differences between pediatric de novo tumors in normal liver, and adult tumors arising in the context of cirrhosis remains unsolved (Dekervel et al, 2014; Weeda et al, 2019). Use of known chemotherapy agents may benefit a smaller group of pediatric HCC and warrants formal prospective study through cooperative group trials (D'Souza et al, 2020). Transplant is contraindicated with metastatic HCC.

Fibrolamellar Hepatocellular Carcinoma

Fibrolamellar HCC (FL-HCC) is more common in females and is primarily a tumor of adolescents and young adults (Allan et al, 2014). In fact, FL-HCC accounts for about 10–25% of HCC in adolescents and young adults, but only 1% of HCC in adults overall (Weeda et al, 2013). There are no known metabolic diseases which predispose to the development of FL-HCC. In adults FL-HCC is felt to have an improved outcome. However, in studies of children this improved survival for FL-HCC was not seen (Darcy et al, 2015).

FL-HCC is typically a solitary lesion with a predilection for the left liver lobe (two-thirds; unusual for hepatic primary tumors) and with well-defined margins and a central scar (70%). FL-HCC shows vascular invasion in up to 35% of cases, frequently metastasizes into locoregional lymph nodes (about 50% of cases), and tends to show unusual spreading patterns, including intraperitoneal spread. A recent discovery of a functional chimeric transcript incorporating *DNAJB1* and *PRKCA* may provide the basis for specific diagnostic and therapeutic strategies (Honeyman et al, 2014).

Hepatocellular Neoplasms-Not Otherwise Specified (HC-NOS), or HB with HCC Features

Hepatoblastoma (HB) with hepatocellular carcinoma (HCC) features are a small subset of malignant liver tumors primarily of children in an intermediate age group (5–10 years) that demonstrate features of both HB and HCC, thereby carrying the "transitional tumor" name. As classically described by Prokurat et al, the tumor cells themselves may have HCC features (Prokurat et al, 2002). Alternatively there may be geographic admixture of histologic patterns with some areas resembling typical HB histology admixed with other areas dominated by more poorly differentiated HCC histology. The children's pathology international consensus recommendation was to classify this heterogenous group as hepatocellular neoplasm-not otherwise specified or HCN-NOS (López-Terrada et al, 2014). Despite this consensus nomenclature, however, it is still quite common to hear them referred to as "transitional" or "HB-with-HCC features." Given the subjective nature of categorizing these tumors, it is difficult to conclude incidence and extrapolate outcome. In the absence of any good prospective data, most centers choose to treat according to either HB or HCC protocols depending upon the dominant tumor type expressed and the quality of the initial response to chemotherapy.

Pediatric Hepatic Sarcomas (Undifferentiated Embryonal Sarcoma, Biliary Rhabdomyosarcoma and Angiosarcoma)

Undifferentiated Embryonal Sarcoma of the liver (UESL)

UESL is a rare malignant tumor of mesenchymal origin, recently shown to share genetic features with the benign mesenchymal harmatoma. Age at diagnosis is usually between 6–10 years but some studies report an older presentation in young teenagers (Techavichit et al, 2016). Patients present with abdominal pain and palpable mass, occasionally fever and anorexia with more advanced cases. Because of their internal myxoid nature, imaging may appear centrally cystic and rupture with biopsy attempts is common. Serum AFP is not elevated. UESL has been reported to arise within mesenchymal hamartomas, and these two tumors share a distinctive karyotype, 19q13.4. (Shehata et al, 2011). In addition, an association with p53 mutations is found in some cases (Sangkhathat et al, 2006). The tumor appears on US as a hetero-echoic mass, and a hypodense multicystic lesion on CT scan or MRI, usually exceeding 10 cm in size, with a predominance for involving the right hepatic lobe (Cao et al, 2014). Metastasis is primarily to the lungs and lymph nodes. Biopsy can be difficult; due to the internal cystic myxoid nature of the tumor, and unguided biopsy attempts often yield nondiagnostic gelatinous material. Best biopsy results are with US guidance to the more solid areas of tumor, or biopsy of a metastatic lesion when present. On resection, most have a solitary large mass that is well demarcated by a pseudocapsule, and on microscopy, the tumors consist of spindle or stellate shaped cells in a myxoid matrix with scattered multinucleated giant cells, and eosinophilic globules (Putra and Ornvold, 2015). The best outcomes are reported when total resection is achieved, either before or after neoadjuvant chemotherapy and followed by multiagent adjuvant chemotherapy (Merli et al, 2015). OLT has been reported in patients with non-metastatic unresectable tumors (Plant et al, 2013).

Biliary Rhabdomyosarcoma

Biliary rhabdomyosarcoma (RMS) is rare accounting for <1% of all RMS in children. It usually occurs in young children (3–4 years of age) (Malkan and Fernandez-Pineda, 2016). This is the liver tumor of childhood that is most likely to present with jaundice, biliary obstruction, and even cholangitis.

Although tumors are often localized at diagnosis, complete surgical resection is rarely possible due to the common hilar location. Biopsy is usually percutaneous, but there are reports by ERCP (Scottoni et al, 2013).

The histopathologic subtype is embryonal or botryoid. Fortunately these tumors are usually very chemotherapy and radiotherapy responsive, and the outcomes have improved with the use of a multimodality approach. Although there are no recent collaborative reports, estimated 5-year survival for patients with local-regional disease has previously been reported at 78%, with metastatic disease being uniformly fatal in the limited number of reported patients (Malkan and Fernandez-Pineda, 2016).

Angiosarcoma

Angiosarcoma is classically found in adults >60 years of age. However, pediatric cases are rarely seen in the context of malignant transformation from infantile hepatic hemangioma (IHH). In fact, type 2 infantile hemangioendothelioma (IHHE) is often considered histopathologically equivalent to angiosarcoma (Dehner, 2004).

The histopathology of pediatric hepatic angiosarcoma is distinct from adult angiosarcoma, because it displays hypercellular whorls of sarcomatous cells, or "kaposiform" spindle cells, in addition to the general features of angiosarcoma (Dimashkieh et al, 2004).

Malignant Rhabdoid Tumor (MRT) of the Liver

Most common in the kidney, the liver is one of several possible primary sites for malignant rhabdoid tumors (MRT) in children. In the kidney it is called rhabdoid tumor of the kidney; in the brain the term is atypical teratoid rhabdoid tumor; and when in the soft tissues or liver as malignant rhabdoid tumor (MRT). MRT can be sporadic or associated with predisposing germline mutations of SMARCB1 (Eaton et al, 2011). INI-1 is consistently negative and there is much speculation that before routine testing for INI-1, many of the "small cell undifferentiated" tumors included in older HB studies, may have actually been MRT. Approximately two thirds of MRT present <1 year of age, 75% of these are metastatic at presentation. Metastases, combined with typical poor response to chemotherapy, result in overall poor survival (only 3% in age <1 year; and 27% in age >1 year of age) (Trobaugh-Lotrario et al, 2011).

Other malignant tumors involving the liver (Nested Stromal Epithelial Tumor, cholangiocarcinoma, hemangioendothelioma,hepatic involvement in hematologic malignancy, metastatic tumors)

Nested stromal epithelial tumor (NSET) of the liver is a rare neoplasm presenting as a solitary hepatic mass, occurring in childhood

and adolescents. Histopathologically, the tumor has nests of epithelioid and spindle cells. Some cases have been associated with ectopic ACTH secretion and rarely even may present with Cushing syndrome (Weeda et al, 2016). It has been reported in one child with Beckwith–Wiedemann syndrome (Malowany et al, 2013). B-catenin mutations (also common in HB) have been reported in two tumors (Assmann et al, 2012). Most patients have a benign course following resection, though tumor recurrence can occur.

Cholangiocarcinoma in adults is associated with choledochal cysts, sclerosing cholangitis and fluke infestation. In children, it is exceedingly rare and has been reported in the setting with biliary cysts, biliary atresia and other biliary tract anomalies (Scheimann et al, 2007).

Infantile Hepatic Hemangioendothelioma (IHEE) is a terminology variably used in the literature on a continuum with infantile hepatic hemangioma (IHH). Two types of IHEE are historically described with type 1 showing a bland architecture and type 2 multinodular that is essentially equivalent with angiosarcoma. Two other types of hemangioendothelioma (HE) should be mentioned: epithelioid HE of the liver is a slow growing vascular tumor that occurs in adults, but that may rarely occur in teenagers. It consists of endothelial cells that histomorphologically resemble epithelial cells but contain novel gene fusion (Errani et al, 2012). Finally, kaposiform HE is a biologically distinct tumor of infants presenting in the first year of life that may involve the retroperitoneum, extremities, neck or chest wall. Isolated liver involvement is not seen; rather, retroperitoneal tumors often expand without regard to anatomic planes and may encase the porta hepatitis and directly invade the liver, pancreas, mesocolon, colon, and kidneys (Croteau et al, 2013). Kaposiform HE is biologically aggressive, and Kasabach-Merritt phenomenon is common with a life threatening coagulopathy and thrombocytopenia.

In the HIV positive patient malignant vascular lesions tend to be more aggressive, more commonly anaplastic and frequently metastatic at presentation. This is commonly a terminal manifestation of severe immunodeficiency and carries a very poor prognosis.

There may be focal or diffuse findings of hepatic involvement in non-solid malignancies. Hodgkin lymphoma involving the liver usually concurrently involves the spleen, and lymph nodes. Liver disease may be focal, but is more commonly diffuse. Nodular lesions are less common, with most having multiple small nodules <1 cm each. They appear hypoechoic on US (Lu et al, 2015). Hepatomegaly is a common presentation in many hematologic malignancies

including hemophagocytic lymphohistiocytosis (HLH), Langerhans cell histiocytosis (LCH) and acute megakaryoblastic leukemia (AMKL), which may occasionally present as an abnormal liver mass; however, they are frequently associated with other signs of bone marrow involvement such as anemia, thrombocytopenia or signs of coagulopathy. Histomorphologic changes and clinical findings in LCH of the liver may resemble primary sclerosing cholangitis or a chronic non-suppurative destructive cholangitis (Shi et al, 2014).

Liver metastases are common in many abdominal solid tumors of children and so non-hepatic malignancies should always be considered in any child with a neoplastic liver process. During the first year of life, liver metastases are often detected in neuroblastoma. Patients with neuroblastoma younger than 12 months of age with metastases limited to liver, skin, and bone (<10% of bone marrow) are described to have the special stage called 4S (or MS) with better outcomes than infants with stage 4 disease (Heij et al, 2008). Other childhood tumors which are known to metastasize to the liver include germ cell tumors, neuroendocrine pancreatic tumors, pancreatoblastoma, gastrointestinal stromal tumor (GIST), desmoplastic small round cell tumor (DSRCT), and Wilms' tumor (WT) (Table 2) (Su et al, 2007; Aronson et al, 2012).

Benign Pediatric Liver Lesions

In children, benign tumors constitute only 30% of liver tumors and most are vascular in origin. Treatment of benign vascular tumors is conservative and seldom surgical (Reynolds, 1999).

Hepatic Mesenchymal Hamartoma

Hepatic mesenchymal hamartoma (HMH) is the second most common benign liver tumor in children after infantile hemangioma. The lesion makes up 8% of all pediatric liver tumors, and 80% of the cases occur within the first 2 years of life. The right lobe of the liver is more frequently involved in the pediatric age group. Prenatal HMH is usually detected by US in the third trimester and has been associated with polyhydramnios, fetal hydrops, intrauterine fetal demise and preterm labor. In neonates, HMH may cause life-threatening abdominal distension and respiratory distress. Recurrent genetic alterations identified in HMH include androgenetic-biparental mosaicism (ABM) and chromosomal rearrangements which result in activation of chromosome 19q microRNA cluster (C19MC). The classic appearance of HMH on ultrasound is a complex cystic mass with internal septations; the cystic portions are anechoic or nearly anechoic with echogenic septa. Histopathologically, HMH is characterized by a lobular growth of myxomatous connective tissue containing scattered

bland stellate-shaped mesenchymal cells. Branching bile ducts similar to ductal plate malformation are also present. Cystic degeneration and extramedullary hematopoiesis are occasionally seen and the larger cystic structures are usually not lined by epithelium (pseudocysts). Entrapped hepatocytes are often identified in the periphery of the lesion. UESL may also arise within HMH or demonstrate focal regions of HMH-like histology UESL may also arise within HMH or demonstrate focal regions of HMH-like histology (Martins-Filho and Putra, 2020)

Hepatocellular Adenoma

Hepatocellular adenomas (HCAs) represent rare, benign liver tumors occurring predominantly in adult females taking oral contraceptives. In children, HCAs comprise less than 5% of hepatic tumors and demonstrate association with various conditions. The contemporary classification of HCAs, based on their distinctive genotypes and clinical phenotypes, includes hepatocyte nuclear factor 1 homeobox alpha-inactivated HCAs, beta-catenin-mutated HCAs, inflammatory HCAs, combined beta-catenin-mutated and inflammatory HCAs, sonic hedgehog-activated HCAs, and unclassified HCAs. In children, there is a lack of literature on the characteristics and distribution of HCA subtypes. Hahn and Putra summarized different HCA subtypes and the clinicopathologic spectrum of HCAs in the pediatric age group (Hahn and Putra, 2020).

Central Hepatic Regenerative Nodules (CHRNs)

Large, regenerative hepatic nodules are seen in approximately 30% of patients with Alagille syndrome. They are thought to be a functional adaptation to vascular changes rather than a neoplastic process. The nodules are typically centrally located, and normal hepatic vasculature coursing through the lesions are noted radiologically. Microscopically, they are characterized by well-circumscribed hepatic lesions with preserved architecture, lesser degrees of fibrosis and relative preservation of interlobular bile ducts compared to the background cirrhotic liver. Regenerative nodules are common in Alagille's syndrome, and should be distinguished from HCCs and adenomas for appropriate management and prognostication (Andrews and Putra, 2021).

Conclusion

Although liver tumors are rare in the pediatric population, they are common in the setting of children with specific risk factors requiring increased awareness and, in some instances, screening. The evaluation of a liver mass in children is largely driven by the age at diagnosis, the presence of any medical comorbidities, and initial testing with AFP and imaging.

Specific guidelines for the diagnosis and management of different tumors have been implemented in recent years such that a multidisciplinary approach is ideal and care should be provided by centers with experience in their management. Pediatric pathologists are the critical members of these multidiciplinary teams.

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Chapter 14

PATIO SYSTEM; NEW APPROACHES IN HATCHING

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Introduction

The tendency of people to broiler meat and layer eggs is probably going to heighten up to about 60% by the coming 20 years globally. According to (Food and Agriculture Organisation, 2010) more than 52 billion broilers were grown in 2010 for meat production. The broiler nurturing accomplishment is subjected via supremacy of zero day old hatchlings. Furthermore, as hatchlings butchery weightiness is gained in a reduced time, the reputation as a good surprise propagates (Romanini *et al.*, 2013). It appears that hatchling excellence and enactment in relationship to survival and growing prospective possibly won't be optimum in existing hatchery supervision techniques. Baby bird trapdoor terminated during 24-36 hours and they are merely removed from the machine when the mainstream of the chickens have crisscrossed the shells, causing an interruption in the first feed and water admittance for a maximum of the hatchlings, and accompanying special belongings on post-hatch development (Gonzales *et al.*, 2003), defence system (Alkie *et al.*, 2019), temperature regulation (Van De Ven *et al.*, 2011a), and gut growth (Geyra et al, 2001). Moreover, weather circumstances possibly will not be best through hatching, and the situation can consequently be interrogated either the hatchlings' necessities can be encountered in existing hatchery systems (Barri *et al.*, 2011). An unconventional hatching scheme is industrialized, called Patio, where hatching and brooding stages are mutual, so that empowering undeviating post-hatch right of entry to feed and water (Van De Ven *et al.*, 2011b). The circumstances in the Patio system vary as those in outmoded hatching systems, e.g. using lesser temperature and relative dampness, air swiftness, the greater bulk of air per egg, and dissimilar egg location throughout crosshatching (De Jong *et al.*, 2019). Patio system possibly will encourage chick eminence, composition, and post-hatch development of chicks. Also, since chickens emerging at dissimilar instants in the hatch booth are bare to nutrients scarcity and a diverse environs for a capricious time, this might reason disparity in biological prestige on the spot of chick assortment (Alaamery and Aldhanki, 2019). Furthermore, the biological reactions of hatchlings just before overdue admittance in water and feed differ with the stage at crosshatching is an issue which wasn't engaged for explanation in the utmost of the preceding training happening about deferred nourishing (Bergoug, Guinebretière, *et al.*, 2013). The old-style hatchery system and interrelated influences on chick excellence and post-hatch enactment are to syndicate the hatching and brooding segments in one arrangement. Throughout the first half decade of the 20th century, patio system for broilers was industrialized in Netherlands. The productive eggs are conveyed from the conservative systems to the patio hatchers after 18th day of the embryo development and are kept there for the last

three days (Giersberg *et al.*, 2021). Subsequently hatchlings persist to stay at this coordinated system throughout their developing age. Consequently, for these hatchlings hatchery handlings, for example, calculating, boxing, and transport are gone astray and chicks take instantaneous post-hatch admittance to feed and water. Correspondingly, the assortment of non-commercial chickens, which are disinterested by hatchery workers next to chick assemblage, is absent in the Patio system as a regular practice after hatch (Maiorka and Dahlke, 2013). Additionally, environmental circumstances in the perinatal stage of Patio fluctuate from the old-styled hatcher storerooms that could impact distinction and post-hatch growth of the hatchlings. Such dissimilar circumstances possibly will have concerns for baby chicken crosshatching at dissimilar instants in the trapdoor booth (Alaamery and Aldhanki, 2019). Merging the hatching and brooding periods empowers abrupt nutrients delivery post-hatch. Additionally, in both hatching schemes hatchlings of dissimilar hatching epochs might react inversely to the initial nutrients uptake (Gonzales *et al.*, 2003).

Ecological circumstances throughout crosshatching in patio system

Zero day old chick excellence and after-hatch development are prejudiced by conservational circumstances during development, and the temperature has frequently remained documented as a life-threatening dynamic. Many documents were issued in book form on weather settings throughout the gestation in hatchery trays, nonetheless, insufficient facts are obtainable on temperature situations (van der Pol *et al.*, 2013). Because of upsurges in eggshell temperature, the egg temperatures in another part of incubation might be greater than stable air temperature and egg temperature of 38-42°C in profit-making incubators stayed cognisant on development days (Molenaar *et al.*, 2011). Distant after special impacts on hatchling superiority and after-hatch development, temperature greater than 38.7°C throughout the late cultivation stage abridged incubation period (Nangsuay *et al.*, 2013) and weightiness of numerous body part (Decuypere *et al.*, 2001). Impacts of lesser egg temperatures (35.4°C) throughout late incubation on chick configuration were incomplete, nevertheless, the development stage increased (Narinç and Aydemir, 2021). In aforementioned researches on the significance of gestation circumstances, hatchlings were scrutinized one or the other at the instant of hatching or during hatchling assemblage from the hatching machine (Yahav *et al.*, 2004).

Consequences of Patio system on embryonic development, weightiness, and survival rate in hatchlings

After chick assortment from the hatcher, supplementary hatchery processes, for example sexing, vaccination, boxing, and transport upsurge the interval while waiting for a settlement in the farm and therefore delay in nutrients admittance for one or two days. If an extended conveyance is convoluted, this epoch might augmented up to three days (Alaamery and Aldhanki, 2019). Under-optimum settings throughout transportation and a postponement in the instant of settlement and the paramount feed and water, consumption is concomitant with more initial transience in hatchlings and diminished development during grower stage of feeding (van de Ven *et al.*, 2009).

Even though, the preliminary days of hatchlings are acknowledged as fundamental for the future growth and weight gain, this is debatable either chickens' biological necessities could be encountered with existing incubation schemes and hatchery supervision dealings (Willemsen *et al.*, 2008). An unconventional arrangement that could hypothetically overwhelm the undesirable possessions of disparity in crosshatching interval and deficiency of feed and water, is a scheme that conglomerates the hatching and brooding periods, in which feed and water can be delivered proximately next to the trapdoor (Bergoug, Burel, *et al.*, 2013). From 2002 to 2006, the same co-ordination was industrialized for chicks. Afterward, this scheme, so-called Patio, was experienced at different three localities in Netherlands during 2005-2008, on the way to appraise significances on hatching capacity and advanced chick development after hatching (Tona *et al.*, 2004).

Time implications on broiler functioning and after hatch development in patio system

An unconventional scheme that can overawe deleterious effects of premature after hatch withdrawal of nutrients, is a scheme which syndicates the crosshatching procedure and after hatch stage, in which nutrients are delivered directly afterward hatch (Barri *et al.*, 2011). During the first decade of 20th century, such planning, baptized Patio1, was established and demonstrated to effectiveness substitute to conventional hatching and brooding organizations, with esteem to hatching capacity, best initial development along with endurance of hatchlings to survive (Wang, 2014). In contradiction of circumstances characteristically perceived in the latter hatching stage, air malaise, comparative dampness, and air swiftness are lesser, while the capacity of air per egg is greater. During preceding stage of incubation, warm air settings may distress growth of numerous tissues,

homeostasis accompanied by body weight (Vyayzenen and Golovey, 2019). About the period immediately before and after birth, chick effectiveness amongst hatching and chick assortment in current two hatching systems, nutrient uptake by hatchlings throughout initial post-hatch time and while waiting for unconcerned from the hatching machine, reckoned, conveyed, then engaged for brooding (van de Ven *et al.*, 2013). This primary epoch of feed and water scarcity was concomitant with greater prompt mortality and compromised after-hatch development (Barri *et al.*, 2011). Another crosshatching scheme was advanced, termed Patio, in which both stages are collective, thus empowering unswerving after hatch entree to feed and water. In preceding trials the situation was exposed that Patio utilities as a crosshatching and growing arrangement, centred on decent hatching of productive eggs and liveability of hatchlings (De Jong *et al.*, 2019; De Oliveira et al., 2008). In an erstwhile training, the bodily processes of fledglings crisscrossed in a hatcher or a Patio system were scrutinized exactly afterward hatching and originated to diverge somewhat among the systems. Nevertheless, great dissimilarity in chick functioning was pragmatic, which was associated with hatching interval within the hatch booth (Van de Ven et al., 2011). The belongings of dissimilar environment settings and instant of paramount nutrient uptake in unadventurous hatchers or the Patio system on hatchling bodily processes in the primary after-hatch stage are unidentified. Because the digestion of hatchlings hatching first or late in the hatch booth appears to fluctuate, they might counter contrarily to initial after-hatch circumstances (Powell, 2016).

Influence of hatching time, primary feed admittance, and hatching schemes on chick development in patio system

The significances of after-hatch abstaining from feed and water on development are undoubtedly not solitary reliant on gap of deprivation, nonetheless might be exaggerated via the atmosphere throughout late gestation and initial after-hatch stage while waiting for hatchling assortment and assemblage. It was presented that more temperature in the course of late incubation decrease development in chicks after-trapdoor (Van De Ven *et al.*, 2012).

Numerous scientists have investigated the characteristics of initial abstinence after hatching and perceived post-screening decision-making and enhanced premature death (Kingston, 1979; Stamps and Andrews, 1995; Halevy et al., 2000; Gonzales et al., 2003). Studies have been conducted on chicks fed straight after placement and chicks in need for an additional 24-48 hours after placement (Dibner et al., 1998; Vieira and Moran, 1999; Halevy et al., 2000; Gonzales et al., 2003).

Broiler productivity until slaughter can only be achieved with first class chicks. It seems that the chick value in terms of development will not be optimum in new incubation systems. Chicks hatch in a 24 to 36 hour period and leave the incubator when most chicks hatch, suspended during feed access with undesirable effects on chick growth. Looking at the climate, the climate during incubation and incubation may not be the best. It could be interrogated whether the broiler chicks' necessities can be met in hatchery exercise. A substitute hatching system was established, named Patio, in which the hatching and brooding phase are shared, therefore allowing straight post hatch contact to feed and water. An unconventional and co-ordinated scheme was developed to address this issue during the late stage of incubation. In the Patio, the airborne heat throughout crosshatching stage stayed established to be 2.8°C lesser while comparative dampness remained around 21.2% lesser paralleled with settings restrained in the conventional hatcher (Uni and Yadgary, 2012).

Based on previous results, it was assumed that typical organ growth could occur in the Patio system between 35.2 and 38.1 ° C, which are typical temperatures maintained at hatching. At this time, Hatcher embryo composition seemed to respond to the enhanced temperatures that appeared in agreement with old knowledge that improved temperatures (> 38.8 ° C) during late incubation could cause lower lung, heart, stomach, liver and intestinal masses during incubation. (Molenaar et al., 2010a; 2011) or heart weights after 21 days of incubation (Wineland et al., 2000; Leksrisompong et al., 2007; Lourens et al., 2007; Molenaar et al., 2011). High temperature effects on hatching body weight, proventricle, liver, gizzard, spleen, and gut weights in broiler chickens appear to be unpredictable among studies (Givisiez et al., 2001; Hulet et al., 2007).

Impact of egg positioning throughout late gestation on crosshatching considerations as well as hatchling superiority in patio system

Fertile eggs are universally hatched for 17-18 days in hatcher trays all through positioning as the big finale of the egg up and around (Butcher and Nilipour, 2009). For the reason that for the duration of this epoch the eggs are rotated habitually at 90° view they repose with their vertical alignment at a 45°perspective (Ayeni *et al.*, 2020). Hatching circumstances in the Patio system vary from in out-dated hatching systems, for example with low temperature, comparative humidity, air speed, and a diverse egg location throughout hatching. Altered circumstances in the Patio system and initial post hatch stage of broiler chickens, particularly initial water and feed admittance, may affect chick excellence, makeup. Besides, chicks hatching at diverse moments in the hatch are unprotected to feed

and water deficiency and a diverse atmosphere for a flexible time, which may lead to a deviation in physical condition of chick assembly from the hatcher. Intended for the preceding 3-4 days of gestation, they are transported to hatching carriers and positioned in hatching machines. In the hatcher carriers, eggs usually place in a straight locus (Van de Ven *et al.*, 2011). In recent times, an unconventional crosshatching scheme was time-honoured, baptized Patio. It is a mutual accommodation arrangement, which was settled to syndicate the hatching and brooding stages (Wilson *et al.*, 2003). Utilizing this modern scheme of hatchery, fertile eggs on the 18th day are not relocated to hatcher carriers; however continue in the horizontal situation in the hatcher trays. Accordingly, as dissimilar to the horizontal location in out-dated crosshatching organizations, eggs in Patio are to be found with the big end up. Furthermore, putting eggs with the small end upright causes inferior chick value after hatch (Elibol and Brake, 2006).

Meat of poultry intake is predictable to rise by 60% in the coming 20 years and will be significant meat group globally till 2030. In 2010, up to 53 billion broilers being the great supplier of poultry industry were raised worldwide. A significant feature for the accomplishment in broiler rearing is the excellence of day old chicks, and with growing chicken meat intake, the plea for day old chicks, currently formed by the hatchery, also grows. Though high value of day old chicks was measured vital to good broiler growth up to slaughter time and chicks come out after 24-36 hours of incubation and are detached from the hatcher whenever, bulk of the chicks has hatched in hatchery. Particularly for the initial chicks hatching will leads to postponements in the initial feed and water access and thus effects negative on chick growth. In the system of substitute hatching, Patio named hatching as well as brooding phase are shared, thus allowing straight post hatch water and feed access. Ecological circumstances in Patio vary from those in hatcheries, which may impact chick eminence, composition, and development. Hatching chicks at altered instants may retort contrarily to diverse situations in both hatching. A different housing for broilers was established, named Patio, in which the brooding and hatching phases are shared. Patio system has different climatic conditions which can be vary from those providing in the hatchers. Hatching had slight effects on hatching makeup and post-hatch expansion was not exaggerated. Since the timing of hatching will greatly affect broiler makeup, it appears significant to take hatching in future educations connected to hatchling structure (Tona et al., 2004; Alkie, *et al.* 2019; Decuypere and Bruggeman, 2007), it appears that chick excellence in terms of growth potential may not be ideal in present incubation systems. Because of two factors, Initially chicks hatch over a time, in about 24 to 36 hours and are

detached from the hatchers when the common of the chicks has hatched (Decuypere et al., 2001). Especially for first hatched chicks, this exercise leads to postponement at the time of settling on the farm and first access to water and feed associated with premature death (Misra and Fanguy, 1978; Kingston, 1979; Fanguy et al., 1980) and post-suboptimal growth mating (Fanguy et al., 1980; Gonzales et al., 2003; Kornasio et al., 2011). Later, situations in the incubator may not be ideal for broiler breeds. In the second stage of incubation, newly hatched chicks give off a significant amount of heat (Tona et al., 2004). In the final stage of incubation, thermal conditions could be affect the growth of multiple organs (Leksrisompong et al., 2007; Molenaar et al., 2010a), thermoregulation (Shinder et al., 2009), and muscle tissue (Piestun et al.). ., 2008).

Conclusion

In a nutshell, hatchlings with primary feed and water admittance in Patio, spectacle heightened biological improvements, bringing about greater weight gains, greater liver glycogen assets, and lesser corticosterone intensities at the instant of hatchling assortment, contrary to feed underprivileged chickens obtained from conventional hatcher. Upgraded after-hatch development in Patio hatchlings paralleled to hatcher chickens is principally owing to former entree to feed and water. Notwithstanding substantial modifications in environmental influences, the inspirations of conventional hatching mechanisms on bodily processes of hatchlings and development efficiency until slaughtering are inadequate. Umbilicus superiority is the furthermost imperative peculiarity of 0-day old hatchling qualitative scores for the reason that this one is the merely characteristic appraised to influence development throughout the whole lifespan. The commonness of lower score chicks is higher in conventional hatching machines rather than Patio mechanism. In the Patio system, the hatching and brooding stages are collectively accomplished, by this means empowering undeviating after-hatch feed and water entree. Environmentally friendly settings in Patio fluctuate from those in hatchers, which can supplementary impact chick excellence, bodily processes, and development. Chickens hatching at dissimilar instants possibly will counter contrarily to these diverse situations in hatching systems. A mutual lodging scheme for broiler hatchlings was technologically advanced, so-called Patio, in which the hatching and brooding stages are joined. Broiler meat production up to final stage could only be attained with great eminence chicks. In terms of viability and progression capacity, chick excellence may not be ideal in existing systems and management procedures. As the chicks hatch in a 24-36 hour window and leave the hatch only when the chicks hatch together, they face the risk of delay and feed intakes associated with bad items. In

count, environmental conditions may not be ideal throughout hatching and in the dated among hatching and chick exclusion from the hatchery. It could so be interrogated either the meat type chicks' necessities could be seen in present hatchery exercise. In a Patio system, temperature and humidity settings diverge from those provided in the hatchers presently in practice. In the Patio scheme, very minor sound effects on chick biological functioning and after-hatch development and survival rate are reported as compared to conventional hatching systems. Since hatching interval exaggerated broiler bodily processes, it is imperative to consider the hatching period into interpretation in forthcoming explorations concerning chick biological processes.

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Chapter 15

5G AND HEALTH CONCERNS

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Electromagnetic pollution

Electromagnetic (EM) pollution has become an important environmental problem worldwide, especially over the last decade. Cell phones, wireless internet providers. and other wireless communication sources are important factors that rapidly increase EM pollution in today's world. Particular attention should be paid to this problem, which is increasing day by day, due to possible adverse health effects (cancer, neurological effects, developmental deficiency effects, etc.) caused by low-intensity long-term exposure in daily life. Research results on the health effects of radio frequency radiation (RFR) emitted by wireless communication sources are of increasing concern to the public every day. RFR associated with various health problems, especially brain tumors, was included in the 2B (possible carcinogen) group by the World Health Organization (WHO) in 2011(Di Ciaula, 2018). With the increase in the number of 5G-based base stations and the Internet of Things (IoT), there will be RFR exposure above the frequencies that have been used extensively to date. Therefore, determining whether 5G-sourced RFR has effects on living things will be a very important scientific contribution toward the elimination of the concerns that have been raised about this issue.

EM Spectrum

The ordering of all EM waves (EMWs) according to their wavelengths, frequencies, or energies creates the EM spectrum (Figure 1). The spectrum, from the lowest frequency to the highest frequency, includes low-frequency, extremely low-frequency, RF, microwave, infrared radiation, visible light, ultraviolet light, X-ray, γ-ray, and cosmic rays.

The wavelength (λ), frequency (f), and energy (E) ranges values determined for RF comprise the following:

$\lambda = 100$ km–300 mm

$f = 3 \times 10^3 – 1 \times 10^9$ Hz

$E = 1.2 \times 10^{-11} – 4 \times 10^{-6}$ eV

RF waves are used in most household appliances (microwave ovens, cordless phones, remote control devices, security systems), communication devices (TV and radio transmitters, mobile phones, radars, Bluetooth, radios, pagers, base stations, etc.), whose usage is becoming more and more widespread day by day, in the medical field (MRI devices, diathermy units, etc.) and workplaces (computers, wireless phones, etc.). In technologically developed countries, people are exposed to RFR more intensely. For this reason, the control of RF fields, their interaction with biological tissues, and their negative effects on human

health have become increasingly important issues in the scientific world, in addition to the public (Hardell & Carlberg, 2020).

Properties of RF-EMWs in mobile communication systems

All types of EM radiation (including visible light) are made up of photon particles and the energy (E) of each photon is proportional to its frequency (f: the number of vibrations per second) and the Plank coefficient (h):

$E = hf = hc/\lambda$

h: Plank coefficient ($h = 6.626 \times 10{-}34$ Joule/s)

λ: wavelength (m)

The wavelength of RF waves can be calculated from the above equation. For example, the wavelength of RFR at a frequency of 60 GHz is 5 mm, and wavelength of 300 GHz RFR is 1 mm. The energies of the RF waves vary between 0.1 and 1.2 meV (milli electron Volt). For example, the energy of 300 GHz of radiation with a wavelength of 1 mm is only 1.24 meV (milli electron Volt) = 0.00124 eV (eV: electron Volt: the smallest energy unit and 1 eV = 1.6×10^{-19} Joules). Since photons require at least 12 eV of energy to extract electrons from atoms or to form ion pairs, waves at the GHz frequency level cannot form ions and therefore enter the non-ionizing radiations section of the spectrum (Bektas & Dasdag, 2017).

Mobile Communication

Mobile communication is a type of communication that can be established with wireless devices. The infrastructure required for this consists of a wireless network with an appropriate number of base stations. Mobile phones are used in the mobile phone system instead of wired phones. Cell phones are devices that send and receive modulated RF signals. Today, mobile phones use signals with a modulation frequency of 217 Hz and carrier frequency of 800–2600 MHz for wireless communication. Mobile phone operators work with a maximum power of 2W (Bektas and Bektas, 2018). Mobile phones communicate with the base station to which the mobile phone network is connected. Voice, messages, or pictures sent by the mobile phone are converted into radio signals and transmitted to the base station. The base station also transfers this information to the phone to be contacted (Bektas & Dasdag, 2017).

Before 5G

The spread of mobile communication to the masses started about 35 years ago in the USA as first generation communication (1G), which brought about the cell structure in voice transmission with Advanced

Mobile Phone System (AMPS) technology. In other words, although the coverage of the base stations was narrowed, the capacity of speaking at the same time was rapidly increased by increasing the number of licensed frequencies in remote cells again and again; however, the transmission infrastructure was still analog. International standards had not yet been established and roaming (international roaming) could not be achieved. Ten years later, due to 2G, the standards and roaming infrastructure that ensure international compliance were established. Then, it was possible to talk about a global mobile network and this network revolutionized mobile data transmission as a result of its digital data transmission possibilities. In this generation, the Global System for Mobile Communications (GSM) technology, established by Europe, achieved leadership all over the world. Voice and data could now travel seamlessly and harmoniously almost anywhere in the world. In 2001, the global success achieved with 3G was built based on Europe's Universal Mobile Telecommunications System (UMTS) standards and the determination of international standards. At this new stage, less than 10 years later, the possibilities of 3G became insufficient to meet the needs of some applications. The technology that defines 4G as a new generation was actually built based on the Long Term Evolution (LTE) (referred to as 4G) and the LTE-Advanced (4.5G) standards that followed it (Ezhilarasan & Dinakaran, 2017). To summarize:

1G: Audio only

2G: Digital voice + SMS

2.5G: Digital voice + SMS + Internet

3G: Digital voice + SMS + Fast Internet + Data

4G: All of the above + maximum connection speed of 100 Mbps on phones, and 1 Gbps on Wi-Fi networks.

On the other hand, RF frequencies used in mobile networks are as follows:

2G: GSM: 850/1900/900/1800 MHz

3G: UMTS: 800–900/1700–2100 MHz

4G: LTE: 700, 1700/2100, 2500–2690 MHz

As can be seen, the frequency value increases in advancing technologies. Each wave pattern contains a mathematical equation and information as a result of that equation, which means that, as the frequency increases, the information transfer increases.

FIFTH GENERATION (5G) TECHNOLOGY

The role of communication in human life increases with digitalization in every new technology. 5G aims to take the parties beyond the usage in the form of "human to human", "human to machine", and "machine to human" in communication technology. The main promise of 5G is to meet the rising needs of mobile services and applications and to provide communication capability to "all objects" by spreading communication technology to all areas of life (BTK, 2018).

International Mobile Telecommunications 2000 (IMT-2000) for 3G systems and IMT-Advanced standards for 4G systems were defined by the International Telecommunication Union – Recommendations (ITU-R). A working group called "IMT-2020 and beyond" was established by the ITU-R to determine the 5G standards. The IMT-2020 vision by ITU-R was published in Recommendation ITU-R M.2083-0 in September 2015 (Series, 2015).

New features that 5G will bring

High speed: This is the amount of data transfer created by downloading and uploading data. In 5G technology, data transmission speed is planned to be 10 Gbps and above. With 5G, with speeds of a gigabit and above, ultra-high (8K) content can be accessed and virtual reality applications can be used. This high-speed technology will pave the way for mobile cloud services that require high speed.

Low latency: Minimizing the latency in data transmission is among the expectations of new technologies. It is planned to realize remote vehicle communication and real-time mobile control applications by keeping the delays in the network below 1 ms with 5G.

High capacity: Capacity is related to the volume of data, and enables a large amount of data to be transferred in bandwidth. With 5G, a channel bandwidth of 100 MHz below 6 GHz and 400 MHz above 6 GHz will be provided. With the high capacity provided by 5G, 100 billion devices will be reached continuously. It will cover a unit area of 1000 times higher capacity than the 4.5 G technology we use today.

Energy efficiency: Efficiency is the ratio of energy used by the system to the energy supplied to the system, aiming to extend the battery life and to do the most work with the energy supplied. Approximately 1000-fold improvements in energy efficiency and battery problems of devices are expected to be reduced. Sensors with a minimum battery life of 10 years are targeted (BTK, 2018).

High geographical coverage and access: Increasing the coverage area with small and many base stations and providing service in a wider geographical area. Uninterrupted connectivity and geographic coverage, mandatory for 5G, will allow for increased economic returns and business plans.

5G is moving forward with a consensus that governments, regulators, and industry will jointly create. The ITU reported that 5G will be built on three basic structures: advanced mobile broadband where high capacity and speed will be provided, massive machine communication where hundreds of billions of machines will communicate, and ultra-security in automation and low delay (Series, 2015).

With 5G technology, new technologies are expected to be implemented in many areas, such as healthcare, transportation, automobiles, agriculture, energy, media and entertainment, smart cities, and smart homes.

In health: Remote healthcare inspection, medical case diagnosis with remote diagnosis and imaging, one surgeon remotely guiding another surgeon via assisted surgery, a surgeon remotely guiding a robot via remote surgery, and health status monitoring with wearable clothing.

In transportation and automobiles: Providing real-time information to the driver via assisted driving, taking over the driver's functions with autonomous/cooperative driving (V2X-vehicle to everything), and driving the vehicle remotely via remote controlled driving.

In aviation: Communication in the military field via drone experience.

In agriculture: Water management, fertilization, animal husbandry, drilling, seeding and spraying, aerial product monitoring, sensors with data-driven agriculture, remote control and control systems, autonomous agricultural machinery and tractors, planting equipment, and synchronous management of all tools and machines that talk to each other.

In energy: Instant monitoring with smart electricity and smart lighting systems, smart cities, smart homes, and smart factories.

In media and entertainment: With the transition from 4K to 8K TV, it is expected to be used in the fields of high resolution, games, security cameras, augmented reality and virtual reality applications.

5G around the world

For the first time in the world, 5G began to be used in the USA, in 2018. The 4 major operators the country began 5G operations in selected important regions at the end of 2019. Asia installed 5G faster than the west; however, China, Japan, and South Korea seem to be leaders in

this regard. The second country in the world to install and use 5G was South Korea. Although 5G installations have been conducted in Germany, Sweden, Estonia, Finland, the Netherlands, France, Russia, and Norway, it has not yet been put into service commercially. In the UK, 4 operators have started to provide 5G service. Switzerland has provided 5G service in 54 cities. Spain had provided 5G service in 15 cities as of November 2019. According to the January 2020 Global Mobile Suppliers Association (GSA) report, 5G is used in 34 countries. In 119 countries, 348 operators are investing in 5G (GSA, 2020).

5G technology will use millimeter waves, which are poorly transmitted by solid material, to transmit the enormous amount of data needed for the IoT. Since the shorter length waves used in 5G technology do not travel very far and are easily blocked, the number of available base stations will not be sufficient. This will require each phone operator to install a base station approximately every 100 m in every urban area in the world (De Grasse, 2016). This will greatly increase RFR exposure. 5G will not only use the 3G and 4G wireless frequencies currently in use, but also add waves of higher frequencies to obtain data at higher speeds. According to estimates, 10 to 20 billion connections (refrigerators, washing machines, security cameras, automobiles, etc.) will be part of the IoT. In every new generation telecommunication device, the amount of information transmitted (speech, text, photo, video, internet, etc.) increases at every moment, which significantly reduces the possibility of living beings adapting to complex signals with higher variability. Rules have been adopted by some countries that allow the effective power of these beams to be up to 20 W, which is 10 times stronger than the levels allowed currently for phones (Pang et al., 2019). Each 5G base station will cover hundreds or thousands of antennas, targeting multiple beams simultaneously on all mobile phones and user devices in the service area (Hong et al., 2017).

Factors Affecting the Absorption of RFRs by Living Organisms

The factors that affect the interaction of RFRs with biological tissues can be classified as physical, biological, and environmental.

A. Physical factors

The type of RF source, frequency of the RFR, its modulation, polarization, power density, near or far field, homogeneity, etc., are important in interactions with biological structures (Gandhi, 1975).

- Frequency of the RFR

RF waves are in the EM spectrum in the range of 3 kHz–300 GHz. The wavelengths of RF waves vary between 100 m and 1 mm. As emphasized earlier, it is a known fact that RFRs do not cause ionization. However, the frequency of RF waves is a very important parameter in their interaction with biological tissues. Living things have a resonance frequency at which they absorb radiation at the highest level, varying according to the polarization of RF waves and the size of the living being. It has been determined that the resonance frequency at which the body absorbs energy most is 4/10 of the wavelength corresponding to the relevant frequency (Challis, 2005).

- RF wave modulation

In broadcasting and communication, the process of converting data, such as sound or image, into electrical signals, using an antenna, by means of EMWs, is called modulation. Since low-frequency waves have a long wavelength, very low-frequency waves, such as sound, are difficult to transport over great distances. Moreover, in order for any variable electrical signal to be propagated through an antenna, the length of the antenna must correspond to of the wavelength of the signal. As a result, in order to transmit a long wavelength signal such as sound, the antenna length must be very large. Assuming that an antenna of appropriate size is used, it is possible that the signals emitted from two opposing stations will be at the same frequency, and because of the interference phenomenon, they will suppress each other. The modulation technique is used to prevent all of these possible negativities, realize data transmission in a safe and practical way, facilitate the propagation of signals, reduce attenuation and noise, connect multiple channels at the same time, and eliminate the limiting effects of devices. In other words, what modulation provides is that a low-frequency signal will be superimposed on a high-frequency carrier signal. Thus, information such as a sound, image, etc., converted into electrical signals, will be encoded (depending on the modulation technique used) and transmitted to the other party via a carrier signal. The signals modulated by the transmitter will be decoded by the receiver via the use of demodulators and then converted back into their original form. In this way, fast, efficient, and economical transmission is provided (Challis, 2005).

- Polarization of the RF wave

If the electric field vector is moving in a single direction while RF is propagating, if it follows a linearly polarized, circular trajectory for the wave, it is defined as a circular polarized, if it follows an elliptical

trajectory, it is defined as an elliptical polarized wave. RF wave polarization is a parameter that affects the absorption level of its energy by living organisms. If the electric field component of RF is parallel to the long axis of the creature, the amount of energy absorbed by the body reaches the highest level. However, the electric field vector of RFRs emitted from the base station and mobile phones is perpendicular to the long axis of the body (Code, 1999).

- Distance from the RF source

The shapes of RF waves, like other EMWs, vary depending on their distance from the source and the objects in the environment. RF waves exhibit a plane wave structure as they move away from the source. The region between the RFR source and the far field, and where the RFR does not show a plane wave structure is defined as the near field. This region can be examined in two sub-groups based on its physical characteristics. The distance shorter than $\lambda / 2\pi$ is considered as reactive near field. Since the E and H field vectors are not perpendicular to each other in this region, it is not possible to calculate the RF field, or to say it in terms of power density. In addition, the wave impedance is different from 377 in this region. The segment between the reactive near field and the far field is referred to as the Fresnel near field region and has many Fresnel points. In this region, the wave impedance is 377 Ω, but the power density is variable depending on the distance (Hitchcock & Patterson, 1995).

Near and far field regions are specified in the safety guides of institutions such as International Commission on Non-Ionizing Radiation Protection (ICNIRP), Institute of Electrical and Electronics Engineers (IEEE), Federal Communication Commission (Federal Communication Commission - FCC) and Telecommunications Authority (TK). However, in these guidelines, λ is defined as the near field and distances from λ are defined as the as the far field (Rappaport, 2002).

The specific absorption rate (SAR) is high in near field exposure and mobile phones, Walkman, dielectric heaters, Bluetooth, radars, etc., are devices that cause exposure to near field EM radiation (Bektas & Dasdag, 2017).

- Power density of the RF Wave

This is the power density on the surface per unit area and perpendicular to the direction of movement of the RFRs. Generally, the energy carried by the EMW is defined by the Poynting vector (S), which represents the magnitude and direction of the EM flux density. It is formulated by the

cross product of the electric field and magnetic field vectors of the 'S' wave.

For a propagating plane wave, the integral of the Poynting vector over any surface expresses the instantaneous power transmitted across that surface and is defined as the power density. Based on the wave impedance equation for plane waves, the power density (S) can be calculated using electric and magnetic field values. Power density is an important parameter in determining the biological effects caused by RFRs in the range of 10–300 GHz (ICNIRP, 1998).

B. Biological factors

The position of biological materials according to the polarization of the EM field (EMF), structural properties, geometry, and size are the parameters that should be taken into account in the interactions between RFRs and biological materials.

- Dielectric constant and conductivity of biological tissue

The dielectric constant of a medium is a coefficient that shows the ability of the medium to store charge. Materials with a large dielectric constant can hold the electric charge longer. As a result, the higher the dielectric constant, the lower the conductivity of the biological tissue, and the lower the dielectric coefficient, the better conductor they become. Each tissue has a unique dielectric coefficient in accordance with its chemical structure. However, the conductivity properties of tissues vary according to the frequency of the EMF to which they are exposed. As the frequency increases, the dielectric constant decreases and the conductivity increases. Biological tissues exhibit much higher dielectric constant, especially at low frequencies, when compared to other homogeneous solids and liquids. This is because biological tissues are made up of different macromolecules, cells, and other membrane-bound substances. As the frequency of the incoming EMW increases, the dielectric constant of the biological material exposed to irradiation decreases, whereas its electrical conductivity increases. In addition to this general tendency, as the frequency of the EMW changes, it is possible to examine the responses of the biological tissue components, such as the molecules, macromolecules, and cell structure, with 4 basic distributions, comprising α, β, δ, and γ (Gandhi, 1975).

The α distribution region: this distribution occurs in the range of 1 Hz–10 kHz and is associated with the movement of ions around the cell membrane.

The β distribution region: this distribution occurs in the range of 10 kHz–100 MHz and is caused by the filling and emptying of cell membranes, acting like a capacitor (the Maxwell-Wagner effect), and the rotation of polar macromolecules and intracellular structures.

The δ distribution region: this distribution occurs in the range of 100 MHz–1 GHz and is due to the hydration of protein-bound water molecules, rotation of amino acids, and partial rotation of the charged side groups of proteins.

The γ distribution region: this distribution occurs in relation to the rotation of electrical dipole moments of water and protein molecules at around 20 GHz.

The electrical properties of biological tissues that change depending on the frequency of the incoming waves are due to the structural properties of water in the tissue and the capacitive structure of the cell membrane. This situation is more pronounced in very low and very high-frequency EMWs. Since the conductivity of water varies greatly at high frequencies, the resistivity of tissues with high water ratio changes very rapidly at frequencies above 1 GHz. In contrast, tissue resistivity changes less at low frequencies (Polk, 1996).

In tissues with high water content, the dielectric constant decreases across cell membranes at high frequencies due to interfacial polarization, and thus, an increase in conductivity occurs. Muscle, skin, the brain, and internal organs have high electrical conductivity due to their high water content. Tissues with low water content, such as fat and bones, have low electrical conductivity.

The cell membrane consists of a double layer of phospholipid layers, consisting of polar head groups and non-polar fatty acid chains. In the electrical circuit equivalent to the cell membrane, the cell membrane corresponds to the inner and outer resistances, and the cell membrane corresponds to the insulator between the capacitor plates. At low frequencies, the RF energy causes the polar head groups in the lipid bilayer to fill and discharge. This results in high capacitance, high relative permeability and the short-circuiting of the RF energy outside of the cell. As the frequency of the RF waves increases, the RF energy cannot be stored by polar molecules in the cell membrane. In this case, because the relative permeability will decrease and cellular conductivity will increase, the RF waves pass through the cell. When the RF waves pass through the cell, heating occurs inside of the cell, due to both absorption and the resistance exhibited by different structures in the cytoplasm (ICNIRP, 1998).

- Reflection, refraction, and diffraction between biological tissue layers

All EMWs undergo reflection and refraction while passing through environments with different impedances (impedance depends on the electrical and magnetic permeability and conductivity of the medium). When passing through sharp corners, they bend (diffraction).

Most of the EMWs are reflected when passing from a tissue with low impedance to a tissue with a much higher impedance. If the impedances of adjacent tissues are equal, the wave completely passes to the other medium.

For example, an RF wave with a frequency of 915 MHz is reflected by 43% in adipose tissue, 73% in the lungs, 78% in muscles, and 79% in blood. When a 433 MHz frequency RF wave passes from one adipose tissue to another adipose tissue, the reflection is 0%, while it passes from the adipose tissue to the blood, the reflection is 56%. This shows that only 44% of a wave with this frequency can pass into the blood (Lin and Michaelson, 2013).

- Penetration of RF waves

The distance in which the RFR coming to the surface of the biological material lose 37% of the initial power density while moving through the tissue is defined as the unsteadiness. The intensity of the RFRs varies according to the dielectric properties of the tissue that they penetrate and the frequency of the RFR.

The energy of the RF wave, which is proportional to its frequency, decreases depending on the resistance the wave encounters while traveling through the tissue. RF waves of the same frequency reach different distances in different tissues. The penetration of RFR is considerably reduced in tissues with high water content. Measurements have shown that the penetration of RF waves in adipose tissue is approximately 5 times greater than in muscle and blood tissues. In addition, as the frequency of the RF wave increases, it becomes difficult to travel through the tissue and thus, its penetration decreases (Lin and Michaelson, 2013).

C. Environmental factors

The temperature and humidity of the environment, the electrical and magnetic field permeability of the materials in the environment, the conductivity of the ground or the environment, etc., can also change the effect of RFR on living things. For example, the reflection of RFR by the substances in the environment, causing scattering or breaking, can change the amount of radiation that living beings are exposed to. In addition, the high temperature and humidity of the environment is a parameter that increases the biological effects of RFR (Polk, 1996).

INTERACTION MECHANISMS BETWEEN RADIOFREQUENCY RADIATION AND BIOLOGICAL TISSUE

The interaction of radiofrequencies with biological material is classified as thermal and non-thermal effects. If the RFR exposed to the biological material causes an increase in temperature in the environment, thermal mechanisms are activated and some heat-sensitive biochemical reactions are affected. However, non-thermal mechanisms are not directly related to temperature increase, but are caused by changes in the electrical and magnetic fields of the RFR on the components of the biological material (Challis, 2005).

1. Thermal mechanisms

It is physically known that RFs do not have enough energy to stimulate cells and cause breaks in DNA. However, reactions occurring in biological tissues that are interacting with RFs may be caused by the increase in temperature caused by the absorbed energy. The electric field components of RFs apply mechanical forces to the electrically charged particles and dipoles in the tissue, while magnetic field components apply mechanical forces to particles with magnetic moment. These forces can cause translation, oscillation, or rotational motion, depending on the mass and mobility of the particle. Thus, the EMF energy transforms into the kinetic energy of the particle. The kinetic energy of the particle is transferred to others through irregular collision (Brownian motion) with other particles. The sum of the kinetic energies of the particles is defined as the heat of the material. Temperature is a physical measure of the average kinetic energy of a single particle, and the increase in temperature is observed with the increase in temperature (Samaras et al., 2015). Absorption of RF energy in biological tissue is related to the electrical conductivity of the tissue. Electrical conductivity is partly due to the translational motion of the charged particles, such as ions. Another factor affecting the conductivity is the rotation of water molecules. Water molecules have a large randomly oriented dipole moment, but when exposed to an electric field, these dipole moments tend towards the field direction. The electric field does a job against the viscosity of the water to rotate the dipoles. The resulting energy conversion results in an increase in temperature in the fluid. This thermal mechanism is effective over a wide range of frequencies, as can be seen from the relation $\omega\tau = 1$, $\omega = 2\pi f = 1$, (ω angular frequency, τ the time taken for the dipole to return to its former direction). Since it is $\tau = 4 \times 10^{-11}$ s for water, it is $\omega\tau \sim 0.25$ at f = 1 GHz. Therefore, this mechanism appears to be quite effective at mobile communication frequencies. The transformation of the RF energy in the environment in biological tissues is

directly proportional to E^2 (E, electric field strength) and the energy loss per unit volume is defined as σE^2 (σ, electrical conductivity) (Challis, 2005).

A photon energy with a frequency of 1 GHz is 4 μeV, and is much less than the energy required to ionize a molecule. Therefore, if an EMW in the RF region causes damage to the biological tissue, it is not possible for this to happen by ionization or excitation resulting from the absorption of a single photon. In the light of this information, it can be said that it is not physically possible for RFR to cause DNA damage. However, research results showing that RFR causes DNA damage should be associated with other biological mechanisms. Moreover, The random movements of charged particles (Brownian motion or thermal noise) that make up biological systems create irregular electric and magnetic fields in the environment. If the applied RFs have a lower energy value than the indoor areas, the system is not affected (Challis, 2005).

Some of the interaction mechanisms of RF and biological materials are based on the stimulation of the vibrations of molecules by the RF energy. Both the energy and momentum must be conserved for this excitation to occur. For energy conservation, the photon (hf_f) energy of the RFR and the phonon energy (hf_s) of the vibration must be equal. In order to achieve this equality, it is sufficient that the frequency of the photon (f_f) and the phonon frequency (f_s) are the same. In momentum conservation, the RF wavelength must be equal to the ultrasonic wavelength. This is possible in optical modes, but they have frequencies considerably greater than 1 GHz, even in very soft tissues such as microtubules. Acoustic modes have frequencies in this range, but momentum conservation is not possible for these modes, because their velocity is 1000 m/s or less, and when compared to the speed of light, they are almost a million times slower. Thus, the momentum of a photon is considerably smaller than the momentum of the acoustic phonon. As a result, molecular vibrations are not stimulated by RFR. However, in complex biological structures such as proteins, it is possible to stimulate some regions with internal degrees of freedom by RFR. After stimulation, these regions return to their former state by emitting phonons (Challis, 2005).

2. Non-thermal mechanisms

a. Change in the protein structure

RFR can cause changes in protein conformation. Proteins consist of sequences of amino acids linked together by peptide bonds. These amino acid chains are in the form of a straight chain or some parts of them are folded or entangled on each other. Therefore, proteins have different potential energy and dipole moments depending on the state of their conformation. The RF electric field can interact with these dipole moments

to change the conformation and thus biological properties of proteins. This situation occurs at low energy values, with temporary increases in local temperatures. However, in high energy values, the temperature increases in question are suppressed by heat shock protein activation. In addition, it has been stated that the local temperature increases that occur for SAR values below the safety limits are too small to cause changes in the protein structure (Laurence, McKenzie, & Foster, 2003).

b. Changes in binding of ligands to cell receptor proteins

The changes that occur during the binding process of light ligands, such as Ca^{+2} to a protein, affect protein conformation and thus, receptor function. The ligand binding to the protein must be seated in a potential well. Chiabrera et al., determined that RF under security limits caused significant changes in the probability of ligand binding by regulating this potential well (Chiabrera, Bianco, Moggia, & Kaufman, 2000).

c. Effects on vibration levels of biological components

Assuming that the vibration frequency of DNA molecules is around 10 GHz, it has been suggested that a microwave with a power of 100 W/m^2 will increase the vibration energy of DNA by approximately 3109 kBT, but this is too low to create significant biological effects (Adair, 2002).

d. Change in electrical attraction between molecules

The arrangement of the moving molecules to form chains in the direction of the electric field in the environment has been defined as the pearl chain effect. It has been determined in vitro that this event is also encountered in erythrocyte and bacteria suspensions under field effect, but it has been suggested that the probability of occurrence in vivo conditions is low. Although blood is the most suitable candidate for the alignment of erythrocytes and other cells in the direction of the applied electric field to observe this effect, the complex and dynamic movement of the blood reduces this possibility. The threshold value required to create the pearl chain effect at frequencies up to 100 MHz depends on the frequency of the RFR, particle or cell size, amplitude, and pulsation of the applied area. Since the oscillation frequencies of high-frequency fields are very high when compared to the oscillation frequency of dipoles, the pearl chain effect is not observed in erythrocyte and bacteria suspensions exposed to high-frequency fields (Adair, 2002).

e. Demodulation of low-frequency electric fields

It is thought that the interaction of pulsed RF signals emitted from biological material and mobile phones is different from the interaction

mechanism with continuous wave RF signals. Pulsed microwaves are known to cause acoustic effects. It is suggested that these effects on hearing are caused by a short-term heating effect by RF pulses with a power of several kW/m^2 or higher. The power of the RFs in question is much higher than the strength of the signals emitted from mobile phones. However, biological effects caused by mobile phones are explained by the demodulation of RFs in biological structures. During the demodulation of RFs, both the initial electric field of the RF and the electric fields created by the digital current and harmonics resulting from the demodulation together create a total effect (Challis, 2005).

f. Free radical formation

Free radicals are highly reactive and therefore short-lived molecules, as they have unpaired electrons. They have an important role in the emergence of various diseases, especially cancer. Studies have shown that low-density RFRs increase the amount of free radicals (Bektas, Dasdag, & Bektas, 2020; S. Dasdag & Akdag, 2016). The spins of the resulting pairs can be positioned as parallel (T) or antiparallel (S), and they oscillate between S and T. If the pairs are in the T state, their probability of reunification decreases and therefore, the probability that free radicals will occur increases. When the couples are in the T position, RFs can extend their coalescence time and therefore, the probability of separation increases, which means an increase in free radical concentration (Challis, 2005).

3. Magnetic field effects

Recent studies have shown that the effect of the magnetic field on tissues is higher than that of the electric field in some special cases. The human body contains magnetite (Fe_3O_4), which is a ferrimagnetic substance. These particles are found in high concentrations, especially in the outermost part of the brain, which is most exposed to RFs during conversation with a mobile phone. The temperature resulting from the interaction of magnetic particles and RFs originating from mobile phones is far below the safety limits. In fact, it has been determined that the magnetic field caused by RF signals emitted from mobile phones is too small to cause ferromagnetic resonance (Kirschvink, 1996). However, it has been suggested that magnetic field pulses originating from GSM mobile phones create a torque on the magnetic particles in the cell membrane and thus cause the activation of ion channels (Dobson & Pierre, 1996).. In one study, it was observed that the cell death rate of bacteria that contained magnetite exposed to 900 MHz RF (GSM) for 8 min increased considerably (Cranfield, Wieser, & Dobson, 2003). Therefore,

it is possible to talk about the existence of an interaction mechanism between the RFs originating from mobile phones and the tissues.

HEALTH EFFECTS OF RFR

Although it is accepted that RFR does not have enough energy to break DNA chains (as much as ionizing radiations), studies conducted in recent years have shown that RFR can cause DNA breaks with different mechanisms. Studies have shown that RFR can disrupt the structures of biomolecules, such as protein, lipids, and DNA, through oxidative stress (Bektas & Dasdag, 2017; Bektas et al., 2020; Lai & Singh, 1996). It has also been found that RFR exposure increases intracellular Ca^{2+} concentration and induces apoptosis. It is a known fact that increased Ca^{2+} input will depolarize the inner mitochondrial membrane by increasing electron transport chain activity and ROS production (Carrasco, Rodriguez, & Pariente, 2015). Widespread use of mobile phones causes adverse effects on many physiological functions, such as memory impairment (Kalafatakis, Bekiaridis-Moschou, Gkioka, & Tsolaki, 2017), increased parasympathetic nerve activity (Misek et al., 2018), increased thyroid function (Baby, Koshy, & Mathew, 2017), weakening of the immune system (El-Gohary & Said, 2017), increased permeability of the blood-brain barrier (Sırav & Seyhan, 2016), changes in amygdala morphology and emotional behavior (Narayanan, Kumar, Potu, Nayak, & Mailankot, 2009), changes in cerebral cortex neurotransmitter release (Kim et al., 2017), cytotoxicity in hippocampal neuronal HT22 cells (Kim et al., 2017), and degenerative changes in the hippocampus pyramidal cells (Hussein, El-Saba, & Galal, 2016). Although the methods and target cells differ in the studies that have been conducted, in general, the current literature shows that the expression of specific genes and proteins in cultured cells and intact animals can be affected by RFR exposure (Obajuluwa et al., 2017).

One of the non-thermal mechanisms caused by RFR is that it causes irregular passages through electrosensitive ion channels on the cell membranes by causing forced oscillations in cellular ions, thus disrupting the electrochemical balance of the cell and causing genetic damage due to free radical release and oxidative stress (Pall, 2013; Yakymenko et al., 2016). In fact, the fact that it causes irregularity in the passages on ion channels is something that the ICNIRP has admitted in its manual (ICNIRP, 2020). Studies conducted on RFR exposure have reported that causes adverse effects, such as heart rhythm disorder (Grigoriev, 2018), disturbed metabolic processes (Volkow et al., 2011), damage to stem cell development (Eghlidospour, Ghanbari, Mortazavi, & Azari, 2017), cancers (Hardell & Carlberg, 2009; Havas, 2017), cardiovascular

diseases (Bandara & Weller, 2017), cognitive and neurological disorders (Deshmukh et al., 2015; Lai, 2018), DNA damage (Bektas et al., 2020; Lai & Singh, 1996; Zothansiama, Zosangzuali, Lalramdinpuii, & Jagetia, 2017), effects on general well-being (Zwamborn et al., 2003), increase in free radical level and oxidative stress (S. Dasdag & M. Z. Akdag, 2016; I. Yakymenko et al., 2016), learning and memory deficits (Narayanan et al., 2009), impaired sperm function and quality (Houston, Nixon, King, De Iuliis, & Aitken, 2016), miscarriage and pregnancy complications (Bektas & Dasdag, 2017), obesity and diabetes (Milham, 2014), etc. Effects on children include autism (Herbert & Sage, 2012), attention deficit and hyperactivity disorder (Divan, Kheifets, Obel, & Olsen, 2008, 2012), and asthma (Li, Chen, & Odouli, 2011).

PROTECTION MEASURES TAKEN BY COUNTRIES

Countries should determine their security limits for the RFR used in communication in accordance with the recommendations of expert organizations such as the WHO and the ITU and to be internationally compliant. Both organizations developed their recommendations based on the ICNIRP guidelines. The international recommended SAR limit value for the general public is 2 W/kg (Table 1) (ICNIRP, 1998, 2020).

Table 1. *Safety limits recommended by ICNIRP (2020).*

Exposure	Frequency Range	Whole body average SAR (W / kg)	Local head/torso SAR (W / kg)	Local limb SAR (W/ kg)	Local S_{tr} (W/m^2)
Occupational	100 kHz – 6 GHz >6 GHz – 300 GHz	0.4 0.4	10 ---	20 ---	--- 100
General Public	100 kHz – 6 GHz >6 GHz – 300 GHz	0,08 0,08	2 ---	4 ---	--- 20

-Whole body average SAR is to be averaged over 30 minutes.

-Local SAR and S_{tr} exposures are to be averaged over 6 minutes.

-Local SAR is to be averaged over a 10 g cubic mass.

-Where relevant, equivalent incident plane wave power density can be used in place of incident plane wave power density.

-Local S_{tr} (Transmitted power density) is to be averaged over a 4 cm^2 (>6-30 GHz) or 1 cm^2 (>30 GHz) square

-"---" indicates that this cell is not relevant to the basic restrictions

To date, 156 countries use this international limit and 19 countries use FCC limit values. Some countries, such as Canada, have combined the safety limits of the ICNIRP and the FCC (GSMA, 2021). The safety limits used by some countries in the world are shown in the Table 2 (WHO, 2017).

Table 2. *The safety limits used by some countries in the world*

		Electric field (V/m)		Power density (W/m^2)		SAR (W/kg)		
Country	Year	900 MHz	1800 MHz	900 MHz	1800 MHz	Whole body	Head and trunk	Limbs
Australia	2017	41,1	58,1	4,5	9	0,08	2	4
Austria	2017	41,25	58,34	4,5	9	0,08	2	4
Brazil	2017	41,25	58,34	4,5	9	0,08	2	4
Bulgaria	2017	6,14	6,14	0,1	0,1			
Canada	2017	32,1	40,7	2,74	4,4	0,08	1,6	4
Finland	2017	41,4	58,55	4,5	9	0,08	2	4
France	2017	41	58	4,5	9	0,08	2	4
Germany	2017	41,25	58	4,5	9	0,08	2	4
Greece	2017	31,9/34,5	45,1/48,8	2,7/3,15	5,4/6,3	0,048/ 0,056/0,08	1,2/ 1,4/2	2,4/ 2,8/4
Iran	2017	41,25	58,34	4,5	9			
Israel	2017	13,0	18,0	0,45	0,9	0,08	2	4
Italy	2017	6/20	6/20	0,1/1,0	0,1/1,0	0,08	2	4
Japan	2017	47,55	61,4	6	10	0,08	2	4
Malaysia	2017	41,25	58,34	4,5	9		2	
Netherlands	2017	41,25	58,34	4,5	9	0,08	2	4
New Zealand	2017	41,25	58,34	4,5	9	0,08	2	4
Norway	2017	41,25	58,34	4,5	9	0,08	2	4
Russian Federation	2017			1	1			
Saudi Arabia	2017	41,25	58,34	4,5	9	0,08	2	4
Sweden	2017	41,25	58,34	4,5	9	0,08	2	4
Switzerland	2017	4/41,25	6/58,34					
Turkey	2017	41,25	58,34	4,5	9	0,08	2	4
United Kingdom of Great Britain and Northern Ireland	2017	41,25	58,34	4,5	9	0,08	2	4
United States of America	2017	47,6	61,4	6	10	0,08	1,6	4
Zambia	2017	41	58	4,5	9	0,08	1,6	4

PROTECTION MEASURES and CONFLICTS

Countries set their own national standards for exposure to EMFs. Most of the national standards are based on guidelines set by the ICNIRP, which publishes guidelines with recommendations on exposure limits that are periodically reviewed and updated as needed. These guidelines cover the non-ionizing radiation frequency range from 0 to 300 GHz. These are based on extensive reviews of all published peer-reviewed literature. Exposure limit values are based on effects related to short-term acute exposure rather than long-term exposure. Because the current scientific data on the effects of long-term exposure are considered to be insufficient to

establish quantitative limits in terms of exposure. International guidelines based on short-term acute effects use approximate exposure limit values or threshold values that could potentially lead to adverse biological effects. Taking into account the measurement uncertainty in science, this lowest threshold value is further lowered to obtain reliable limit values for human exposure. For example, the ICNIRP uses a reduction factor of 10 to achieve occupational exposure limits for workers and it uses about 50 to achieve exposure limits for the general population. These limit values vary according to frequency and are different for low-frequency areas and high-frequency areas.

On the other hand, most people, at home and at work are exposed to a mixture of weak electric and magnetic fields from the use of household appliances and industrial equipment, as well as the transmission of electricity, telecommunications, and broadcasts. Even if there is no external electric field, tiny electric currents are present in the human body due to chemical reactions that are part of normal body functions. The way EMFs affect health depends on the frequency, strength, and duration of exposure. The frequency of the applied field is also important in terms of the results of the effect because different frequencies interact with the body in different ways, and the effects of low-frequency fields are not the same as those produced by higher frequencies. Low-frequency fields cause nerves and muscles to be stimulated, while high-frequency fields cause tissues to heat.

Moreover, the ICNIRP limits were established by considering the thermal and acute (short-term) effects, and there are massive non-thermal effects. Studies on long-term exposure effects are still ongoing. Current limits have been developed for healthy individuals. The person defined as a healthy person is a male who weighs 70 kg and is 1.7 m tall. However, there are sick people in society, there are extremely sensitive people, there are women, there are pregnant women, and there are babies and children. What the exposure of children will be over the long term is unknown, because they begin to be exposed at the age of zero. Child and adult brains are very different. The skulls of children are thinner; hence, it is more affected by RF.

The interaction of RFs at the molecular level is not thermal in nature. RFs apply an electrical or magnetic force to the particles that it comes into contact with. As mentioned above, the forces that the particles are exposed to cause an increase in kinetic energy at the microscopic level, and heat is released as a result of this mechanism. Physiological and biochemical events that occur at the macroscopic level are related to the temperature level that occurs. Therefore, a threshold value is required to distinguish thermal and non-thermal effects in biological processes. On

the other hand, it should not be forgotten that the amplitude or intensity of the EMF is also important in the emergence of thermal effects.

Consequently, it is misleading to claim that the effects observed under safety limits are not thermal (Bortkiewicz, 2019). The time-varying areas above 100 kHz, if they are strong enough, cause a significant increase in temperature in the tissues they penetrate. In the case of prolonged or intense exposure, thermoregulatory mechanisms are activated to distribute the absorbed energy, and if they are insufficient, local or general body temperature increase may occur.

Numerous recent scientific publications have shown that the RF-EMF affects living organisms, even at levels that are well below most established international and national safety limits. Adverse health effects caused by RFR include an increased risk of cancer, cellular stress, increased harmful free radicals, genetic damage, structural and functional changes in the reproductive system, learning and memory deficiencies, neurological disorders, and the deterioration of general well-being in humans. The aforementioned damage by RFR go beyond the human race and also threaten plant and animal life. The ICNIRP is one of the most effective organizations that determines the safety limits for exposure to RFR. The ICNIRP only accepts the thermal effects of RFR and determines the safety limits of exposure to RFR by reference to EMF values that may cause adverse health effects and temperature increases. As a result, the results of large-scale and numerous studies on the non-thermal effects of RFR are ignored.

Updated security limits for RF-EMFs (including 5G) were recently published by the ICNIRP on March 11, 2020 (ICNIRP, 2020). However, the results of many experimental studies showing various non-thermal biological/health effects of RFR (Belpomme, Hardell, Belyaev, Burgio, & Carpenter, 2018; Miller, Morgan, Udasin, & Davis, 2018), as in previous guidelines (ICNIRP, 1998, 2009), have not been taken into account. Even in the guidelines published by the ICNIRP in 2020, it is seen in Table 3 that the limit values for RFs for the general public are quite similar to those in 1998. In addition, the only mechanism taken into account by the ICNIRP in determining the safety limit values is the temperature increase, and it is also possible that the temperature increase will occur with the exposure to RFR used by 5G. The majority of cellular responses and various health effects, such as DNA damage, protein damage, chromosome damage, and reproductive declines, are not accompanied by a significant temperature increase, and the effect of EMFs on living things is well-known by scientists (Dasdag & Akdag, 2016; Dasdag et al., 2012; Lai, 2018; Yakymenko et al., 2016).

Table 3. *Comparison of ICNIRP 2020 and 1998 safety limits*

Exposure	Frequency Range	E-field strength (V / m)		H-field strength (A / m)		Incident plane wave power density (S_{inc}) (W / m^2)	
		2020	1998	2020	1998	2020	1998
Occupational	>30-400 MHz[#]	61	61	0,16	0.16	10	10
	>400-2.000MHz[*]	$3\ f^{0.5}$	$3\ f^{0.5}$	$0{,}008\ f^{0.5}$	$0{,}008\ f^{0.5}$	f/40	f/40
	>2-300 GHz[*]	----	137	----	0,36	50	50
General Public	>30-400 MHz[#]	28	28	0,073	0.073	2	2
	>400-2.000 MHz[*]	$1{,}375f^{0.5}$	$1{,}375f^{0.5}$	$0{,}0037f^{0.5}$	$0{,}0037f^{0.5}$	f/200	f/200
	>2-300 GHz[*]	----	61	----	0,16	10	10

-f is frequency in MHz.

-S_{inc}, E^2 and H^2 are to be averaged over 30 minutes, over the whole body space. E- and H field values are to be derived from these averaged values.

-For frequencies up to 2 GHz, compliance is demonstrated if either the E-field, H-field or S_{inc} value is within the reference levels; only one is required.

-"---" indicates that this cell is not relevant to the reference levels.

-[#]. For frequencies up to 400 MHz: For reactive and radiative near-field exposure conditions, exposure is compliant with the reference levels if both E- and H-field levels are within the relevant far-field reference levels

-[*]. For frequencies above 400 MHz: Far-field reference levels are also applicable to radiative near-field exposure conditions; no reference level is provided for reactive near-field exposure conditions.

Moreover, the WHO's International Agency for Research on Cancer concluded in 2011 that RFR at frequencies of 30 kHz–300 GHz can cause cancer (Group 2B) in humans (Deruelle, 2020). However, recent findings, including recent studies on cell phone use and brain cancer risks, have shown that RFR is carcinogenic to humans (Carlberg & Hardell, 2017). RFR is now classified as a Group 1 carcinogen, along with tobacco smoke and asbestos.

However, at the end of a detailed technical report that was published by Swedish researchers, wherein the results of live (vivo) and laboratory (in vitro) studies, conducted by numerous researchers, of frequencies ranging between 6 and 100 GHz (including 5G) were reviewed with regards to the effects of RFR at mobile communication frequencies on the body, it was stated that there were no consistent results and no other scientifically valid or proven effect has been proven, except for the thermal effects (Simkó & Mattsson, 2019). On the other hand, scientific studies that have been examined by more than 10,000 scientists have shown the harmful effects of RFR on human health (Group, Sage, & Carpenter, 2012).

REACTIONS AND RECOMMENDATIONS

Stakeholders, industry, and governments have thus far been involved in the development of 5G. However, the well-known international scientists who have proved the biological effects of RFR on living things in thousands of studies have been excluded (Hardell, 2017). Current safety rules are based on the outdated hypothesis that warming is the only harmful effect of EMFs. As Markov and Grigoriev stated, "Today standards do not take into account the real pollution of the environment with non-ionizing radiation" (Markov and Grigoriev, 2013). Hundreds of researchers, including many scientists supporting to this appeal, have documented that many different types of acute and chronic diseases are induced by RFR levels far below international guidelines non-thermal (Blank, Havas, Kelley, Lai, & Moskowitz, 2015). Biological effects occur even at near-zero power levels. The RF signals used in 5G are pulse modulated, as in other technologies. The adverse health effects results from both high-frequency carrier waves and low-frequency pulses (Blackman, 2007).

To protect against non-thermal effects, the exposure time should be taken into account. 5G will expose everyone more and more, day and night, simultaneously, and continuously. New safety standards are needed and must be based on cumulative exposure, and not only power levels but also frequency, bandwidth, modulation, waveform, pulse width, and other biologically important characteristics. Antennas should be limited to specific, publicly determined locations. To protect people, antennas should be excluded from areas with public right of way. To protect wildlife and nature, it must be removed from wildlife sanctuaries and minimized strictly in remote areas of the Earth. To protect all living things, commercial communication satellites should be limited in number and banned in low- and mid-Earth orbit.

Today, a large part of the world's population is regularly exposed to levels of RFR that cannot be measured close to the body. Moreover, the rapidly developing brains of young children are regularly irradiated with RFR emitted by these wireless communication devices for hours every day. Researchers who have more than a thousand publications published in serious international journals on the subject and citizens with concerns about the subject are running a campaign urgently inviting the WHO to form an independent "global commission". The main purpose of the commission will be "EMFs used in wireless communication and their effects on health" and "To determine new safety limits based on biological foundations".

On the subject, a moratorium application signed by more than 390 international scientists and medical doctors was submitted to the

European Union (EU) in September 2017; however, no response has yet been received from the EU (Hardell & Carlberg, 2020). The Bioinitiative Report, REFLEX Project Report, Interphone Project Report, National Toxicology Program Report (Hardell & Carlberg, 2019; Smith-Roe et al., 2020), all list diseases including cancer and other risks. related to RFR. Additional reports, such as the Ramazzini Report, are the result of high-quality studies by scientists, without conflict of interest (Hardell, 2017). It has been demonstrated that these adverse health effects can occur at levels that are far below the radio frequency EMF exposure limits recommended by the ICNIRP and approved by the WHO's Electromagnetic Fields Project to prevent sudden, short-term temperature increases. It has been stated that guidelines of the ICNIRP may have serious consequences for humanity and the environment (Hardell, 2017; Hardell & Carlberg, 2019; Smith-Roe et al., 2020).

Moreover, in the last few years, several important calls have been made by expert scientific committees to reduce exposure limits to EMF and the risks associated with them, alerting public health authorities to the issue by providing evidence and suggestions for solutions. These calls include the Freiburg Call Salzburg Decision, Catania Decision, Benevento Decision, Venice Decision, Porto Alegre Decision, Copenhagen Decision, American Academy of Pediatrics recommendations, Seletun Scientific Statement, International EMF Call of Scientists, the 5G Call, 2020 UK and International Medical and Scientific Experts and Practitioners Ionizing Statement of Consensus on Health Effects of Non-Radiation, and many other important documents.

If WLAN, DECT, Laptop, and mobile phones are close to the body, their SAR values vary between approximately 1 and 0.0001 W/kg. In the case of using mobile phones with headphones, it was found that the initial radiation effect remained between 1/8 and 1/20, although it resists the ear. When using a Bluetooth headset, the SAR values are reduced by at least 1/10 (Atakan, 2020).

As a result, it was stated by scientists working in this field that 2G, 3G, 4G, and Wi-Fi are not secure, and 5G should be evaluated more carefully in terms of acceptable harmful biological effects.

At least, as the general public, we can take the following measures:

-Speak succinctly when talking on a cell phone, try to keep it at least 2.54 cm away from your head, talk through the loudspeaker, use a headset, and prefer to text.

-Children and pregnant women should avoid excessive use of mobile phones and other wireless devices, as they are more vulnerable.

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Chapter 16

THE BASICS OF NUTRITION AND DIGESTION AND THE SPECIFIC NEEDS OF ATHLETES

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The Basics of Nutrition and Digestion and the Specific Needs of Athletes

For the body to function and perform even the most basic of daily tasks it needs fuel from the three primary macronutrients; fat, protein and carbohydrates, as well as numerous micro-nutrients. For those wanting to develop and maintain a healthy body, fueling it requires more careful consideration of the body's nutritional needs, and for athletes, detailed planning is needed to make sure the body is getting exactly what it needs to function at its best. Once a plan for a healthy balanced diet which meets the individuals PDI'S (performance daily intakes) has been established, the ability to process the food with a healthy digestive system able to absorb and utilize the nutrients, is essential in maintaining overall health and wellbeing, and achieving optimum athletic performance.

Introduction to the Primary Macronutrients: Carbohydrates, Fat and Protein

At the core of any diet are three primary macronutrients; carbohydrates, fat, and protein, all of which the body needs to function.

Carbohydrates

For a healthy balanced diet, carbohydrates will make up the majority of daily calories. They provide the whole body with energy, from the muscles to the brain cells, in the form of glucose (sugar), and as carbohydrates are consumed the body receives the energy immediately (Leal, 2020). Though low carbohydrate diets are popular, they are the body's main fuel source, the quality and source of the carbohydrate is therefore what is important.

Carbohydrates can be categorized into:

Simple Carbohydrates

Simple Carbohydrates can be found naturally in milk and fruits and vegetables, though they are found most often and in the highest percentages in processed foods, having been added during production.

Sugars: These are simple carbohydrates found naturally in fruits and vegetables. The AHA (American Health Association) recommends a daily limit of 24g of sugar, an apple having around 10g (*Added Sugar*, n.d.). Products such as soda and deserts will have added sugars, these should be avoided in order to maintain healthy blood sugar levels and avoid empty calories, the body takes no nutritional benefit from sugar, natural or artificial.

Complex Carbohydrates

Starches: Starch is a solid white substance made up of long chains of sugar molecules that take the body longer to digest, releasing steadily into the blood stream avoiding fluctuations in blood glucose levels (Byrne & Yu, n.d.). Starchy vegetables include pumpkin, corn and potatoes, and starch can also be found in cereals and wholegrains.

Fiber: After the digestive system removes nutrients from food and breaks it down, fiber is what is left over. The body cannot breakdown fiber and so it provides roughage and bulk to move harmful carcinogens through the system and ensure regular bowl movements keeping the digestive tract clean (*High-fiber Foods*, n.d.). Fiber is not found in meat or dairy products and can be found in the highest quantities in natural unprocessed foods, namely fruits and vegetables. Ultra-processed and "white-foods" like bread and pasta are often stripped of the natural fiber they contain.

Fiber can be categorized into:

Soluble: Dissolves in water, becoming a gel like substance that helps the body control blood sugar and manage cholesterol levels (Healthline).

Insoluble: This is not dissolved when combined with water and so helps avoid constipation, it is found in whole grains, and many vegetables such as celery.

Fats

Fats are a three-molecule structure, a triglyceride, referred to as fatty acids, fats or lipids. The human body naturally produces most of the fat it requires, however, essential fatty acids omega-3 and omega 6 must be taken from food sources. Fat is an essential part of a healthy diet, slowing digestion keeping us full for longer, providing essential energy reserves, adding flavor to food and providing an essential source of calories to those unable or unwilling to eat large quantities such as children, the elderly and the sick, and increasingly commonly endurance athletes. Though fats have been demonized by the food and diet industry over the past decades and overly processed diet and low-fat options sold as alternatives, our bodies need fats to work. The thing to know is the difference between the types of fat and thus ensuring quality fats are being consumed in moderate amounts, over excessive quantities of harmful fats.

Fats can be categorized into:

- ***Polyunsaturated*****:** These are associated with healthy cholesterol levels, though should be eaten in small amounts, a maximum of 10% of

daily calories should come from polyunsaturated fatty acids. They can be found in oily fish, flaxseeds and soya oil.

- ***Monounsaturated:*** These are healthy, naturally occurring fats, liquid at room temperature and found in olive oil, avocados and nuts and seeds such as cashews. Up to 20% of daily caloric intake can be made up of these fats.

- ***Natural Trans Fats (ruminant):*** These make up about 2-6% of the fat in dairy products and 3-9% of the fat in cuts of meat, they contain conjugated linoleic acid which is available as a dietary supplement. This form of fatty acid is considered healthy if eaten in only low to moderate amounts. Even natural trans fats should make up less than 1% of daily calories (maximum 2g).

- ***Artificial Trans Fats***: These are found in ultra-processed foods such as pizza, baked goods like pies and vegetable spreads, they are best avoided as they have been linked to severe health issues such as heart disease and high cholesterol. They are often formed when foods are processed to extend shelf-life.

- ***Saturated Fats***: Occur naturally in foods, mainly meat and dairy, though it can also be found (without the cholesterol) in palm oil and coconut oil. The AHA suggests staying below 13g per day, no higher than 6% of the daily caloric intake.

Protein

Proteins, long chains of 20+ amino acids, are found in all living things and vary between species and even between organs (Haurowitz et al., 2020). The amount of protein recommended per day is related to weight, 0.8 grams per 1 kilo is the suggested amount from the National Academy of Medicine (*Protein*, n.d.). The body cannot store amino acids as it does with fats and carbohydrates and it does not produce its own essential amino acids, so daily intake is a must (Oz, n.d.). The amount of protein intake, however, can vary greatly depending on the diet being followed, the source of the protein being the most important consideration.

Proteins can be categorized into:

Complete Proteins: The body is unable to produce certain essential amino acids on its own so gets them from food sources. Proteins that have all of these nine essential amino acids are considered complete proteins (*Do I Need*, 2019). Complete proteins can be found in fish (Atlantic salmon 19g per 3 oz), beef (22g per 3 oz), cheese (cheddar 21g per 3 oz), and whole sources of soy such as tempeh (15g per 3 oz), edamame (17g per cup), and tofu (7g per 3 oz). Taking even complete protein from

a variety of sources is recommended as each has different benefits for the body, for example protein from fish has been found to have a higher satiating effect than other complete proteins, and dairy most efficiently aids in muscle protein synthesis (Gilbert et al., 2011, pp. 16-31).

Incomplete Proteins: Incomplete proteins lack one or more of the essential amino acids needed for cell regeneration (*Incomplete Proteins*, n.d.). Combining incomplete proteins is part of nutritional planning for most plant-based diets to provide variety outside of whole soy and quinoa (complete proteins), for example wheat bread has 13g of protein per 100g, which contains five of the essential amino acids, eaten with peanut butter (25g per 100g) it makes up the total nine essential amino acids (McDermott & Pace, 1957, pp. 446 - 452).

Meeting the Bodies Nutritional Needs

The recommended daily intake of macronutrients (carbohydrates, fat and protein) is often based on a standard level of physical activity and demands on the body, with the aim of maintaining health. When planning a balanced diet for athletes, the bodies needs vary depending on individuals' performance goals, so it is worth looking at the three E's of nutrition and how these connect to Performance Daily Intake (PDI).

The Three E's of Nutrition and PDI

The three E's of nutrition are; essential nutrition for survival and basic health, nutrition for optimal health and nutrition for athletic performance. The 1st category covers nutrients needed for basic survival, physical and mental development, productivity, and maintaining general well-being throughout life by avoiding illness connected to malnutrition (Gillaspy, n.d.).

While the E related to optimal health represents a diet rich in all nutrients and in optimal (ODA) rather than basic quantities as in the first, and would include a diet personalized to suit an individual. There are many benefits to having a personal trainer or coach and the knowledge and guidance to match physical activity with nutritional needs is probably one of the biggest (*15 Benefits*, n.d.).

The Essential nutrition for athletic performance focuses on the greater needs of those expending large numbers of calories, and who need to repair muscle damage caused by above average physical activity.

It is often the case that an individual takes in too much of a select few nutrients while neglecting others, even following guidelines related to how much of each macro and micronutrient should be consumed, the

recommendations are based on the first category, they do not consider different body types, demands on the body or varying routines.

PDI (performance daily intake) is utilized in the 3rd of the 3 E's: Athletic performance. The nutritional intake of an athlete, including nutrients taken from both food and supplement sources (food alone would likely not provide enough nutrients as PDI's would generally be needed in higher amounts than most RDA values) should be based on the science of sports and fitness nutrition and be incorporated into a personalized diet plan. PDI guidelines cover a variety of body types; male/ female, shape and size and varying levels of activity, but are intended for healthy, active adults. PDI aims to provide for the metabolic needs of an individual athlete. This cannot be static as the cycle in which an athlete trains is not, meaning that at different points in the cycle the nutritional needs for optimum performance/recovery will vary, for example on competition days. Therefore, planning must be as accurate as possible considering all planned exercise expenditure, spontaneous physical activity and non-exercise activity (Thomas et al., 2016, p. 543).

General Macronutrient Recommendations and the Influence of Body Type, Physical Health, and Performance Goals.

As stated previously, with reference to a few general RDA (recommended daily amount) guidelines, the three macronutrients are fats, carbohydrates and proteins. Carbohydrates, when broken down, become glucose, a sugar essential for providing the body with energy. Fats aid the absorption of essential fat-soluble vitamins (A, D, E, K) into the body, help stabilize hormones, are a source of energy, and provide the body insulation. Protein regulates the metabolism while building and repairing cells and tissue: A planned and monitored intake of all three groups dependent on the current specific demands of an athlete is essential to maintaining optimal health and athletic performance.

The general RDA for the macro-groups is 45-65% carbohydrates, 20-35% fats and 10-35% proteins. The exact percentage will vary depending on an individual's body type as well as other demographic factors such as age and gender, and the individual's performance goals. Medical conditions should also be considered.

Metabolic Body Types

There are three metabolic body types to consider when assigning macronutrient percentages; ectomorph (narrow frame, weight gain challenging), mesomorph (athletic or muscular with significant lean mass) and endomorph (large frame with higher amounts of muscle and fat). The

requirements of each body type can be met by working out the appropriate macros.

Ectomorph: 55% carbohydrates, 25% protein and 20% fat

Mesomorph: 40% carbohydrates, 30% protein and 30% fat

Endomorph: 25% carbohydrates, 35% protein and 40% fat

Allowing for adjustment for blood pressure and cardiovascular concerns as well as weight goals.

Another core consideration when planning for the appropriate intake of macro groups is activity level; frequency, time, and intensity. A sprinter (anaerobic exercise) using high energy bursts will burn through a higher number of calories from carbohydrates than a marathon runner (aerobic) whose energy levels need sustaining over extended periods. Though both aerobic and anaerobic exercise utilize carbohydrates as fuel at the start of an activity, the body switches to burning fat during aerobic activity. Neither type of exercise uses protein as a source of energy, though protein is essential in balancing blood sugar during exercise and for athletes repairing muscle damage after finishing (an athlete would need 1.4-1.8 grams per body weight of protein, a regular person needing only around 0.8). If activity levels increase the fat/carbohydrate level would need to be adjusted accordingly to provide the additional calories and ensure muscle glycogen is enough.

Common Conditions Affecting Nutritional Needs

There several commonly known conditions that will influence the bodies nutritional needs and also the possible need for elimination or reduction of certain food groups. Two will be shown here:

Diabetes

In a drastic shift from exercise being considered a danger for diabetics due to metabolic risk, it is now considered a treatment for the condition, though the issue of safe glycemic control remains (Novials & Murillo, 2012). An athlete with diabetes will undergo the same intense training regime as any other athlete, and so the excess demand for energy when training and competing will have to be met (Hornsby & Chetlin, 2005). The recommended calories from carbohydrates for athletes is generally 55-60%, this would typically be reduced to no more than 50% for diabetics (*Nutrition for Athletes*, 2020). The need to anticipate and manage glucose response will need careful planning and nutritionists/coaches /trainers should always consider the Glycemic Index (GI) when planning any changes to a diabetic athlete's diet in order to reduce potential negative effects. Foods higher on the Glycemic Index increase blood sugar, and so

carbohydrate intake, for example, may need limiting accordingly, though reducing carbohydrate levels without adjusting insulin dosage will lead to hypoglycemia (*What Is a Healthy*, n.d.).

Balancing diet, medication and physical activity is a challenge for the diabetic athlete.

There are instances where the condition is a result of the extreme diet followed to achieve the athlete's goals. Diabetes was found to be present in athletes, especially female, who take part in endurance sports and aesthetic sports such as gymnastics, and has been attributed to extreme weight control (Hornsby & Chetlin, 2005).

High Cholesterol

High cholesterol is an excess fatty substance found in the bloodstream most commonly caused by unhealthy lifestyle choices such as consumption of fatty foods, smoking, drinking alcohol and lack of exercise. Increased bad cholesterol (LDL) is associated especially with the consumption of excess trans-fats, so it seems a paradox that elite athletes such as Michael Spitz, a former Olympic swimmer, would suffer from high cholesterol (Schneiderman, 2008). High cholesterol is an issue now found in many endurance athletes, blamed mainly on the current popularity of low carbohydrate, high fat diets (Creighton BC, et al., 2018).

Trans fats are generally associated with processed foods; however, they are also found naturally in dairy and meat, and high protein foods such as whole eggs are staples of low-carbohydrate diets such as Atkins.

The Differences Between Micronutrients and Macronutrients

Moth macronutrients and micronutrients are essential to maintaining a healthy body and are sourced from the food, drink and supplements we consume. The only difference between them is the quantity the body requires. Micronutrients, referring to those needed in small quantities such as vitamins and minerals, and macronutrients referring to the core caloric, energy providing nutrient groups (fat/carbs/protein) needed in larger amounts. Both are essential to keeping the body healthy overtime.

Micronutrients most important role is developing and maintaining a strong immune system and enabling the body to recover and repair. Damage to tissue and muscle is common for those undertaking regular intensive workouts, so the ability for the body to recover quickly is essential. Though anyone including exercise in their regular routine will lose micronutrients at a faster rate so will require an increase in micronutrient intake. For athletes, the maintenance of lean body mass would be impossible without meeting the required amounts of micronutrients.

Micro-nutrient Deficiencies in Athletes

The increased demand placed on the body by an athlete's schedule can often lead to a deficiency in certain micronutrients.

Iron deficiency among female athletes is likely the most common micronutrient deficiency, affecting up to 20% of all women (varies between ethnicities) compared to 2% of men, it is a common issue. For female athletes' menstruation can further increase the deficiency in iron leading to fatigue and weaker athletic performance.

A vitamin D deficiency is very common among athletes and non-athletes. Vitamin D though produced naturaly by the body, can only occur during exposure to the sun. The local climate, lack of outdoor activity, even the wearing of strong sunscreens can restrict the amount of sun exposure the body receives. Without vitamin D the body's immune system is unable to remain healthy and strong along with zinc, which is also often found lacking.

Lack of vitamin D also inhibits the body's ability to absorb calcium, adding to another common deficiency. The RDA for calcium is 1000 mg, this can increase for those who sweat a lot from high-intensity workouts.

Post workout drinks now commonly include electrolytes, this is to help with the additional 10-20% RDA of magnesium needed by athletes to help them relax muscles and reduce inflammation, and also provide potassium which the body needs to hydrate. Electrolytes are lost when an athlete sweats during workouts, so need replacing or issues like cramping can occur. Including magnesium and potassium in post workout meals would be beneficial.

Digestion

What We Eat

Ensuring the body is taking in enough prebiotics and probiotics is essential to maintaining a healthy digestive tract. At the same time avoiding food and drinks which will upset our digestive rhythm.

Eat

- Fermented food such as miso and kimchi. These have probiotic bacteria and also many vitamins and minerals.
- Foods with fatty acids and polyphenols like olive oil.
- Foods high in fiber (soluble and insoluble), which gut bacteria needs, like peas and bananas. Protein rich foods such as fish take longer to go through the system so should be eaten in moderation.

- Foods which reduce or control harmful bacteria like garlic and sprouts.
- Foods which help to maintain a healthy digestive rhythm such as ginger.

Avoid

- Wheat based products and glutenous grains like seiten, as they can increase gut permeability leading to issues like 'leaky gut' where the spaces in the intestine walls widen allowing more harmful bacteria through.
- Processed foods often have low fiber content and so can be difficult to move through the digestive tract.
- Dairy, though our bodies produce lactase which allows us to digest dairy, as people age they produce less and less and can become intolerant to dairy over time and unable to easily digest it.
- Refined oils (corn, soy, canola etc.) are processed with chemicals that can disrupt the digestive tract such as acids, alkali or bleach, this process leads to PUFas (rancid polyunsaturated fatty acids).

The quantity and quality of the food consumed is also vital to maintaining a healthy system, eating too much, even of healthy whole—foods will put the system under strain and can result in feeling sluggish.

Factors Influencing Digestion

When looking into a person's digestive health, gut health, food quality, and the effects of work are all areas to be considered.

Gut Health

Having a healthy balance of gut bacteria is not just essential to a healthy digestive tract, if the gut microbiome is imbalanced it can have a negative impact on many areas of health including a weakened immune system, high cholesterol and weight gain. There are trillions of kinds of bacteria in the human gut, so maintaining a balance of healthy microbes is not always easy, especially as every person has a unique microbiota: Partly inherited from the mother, the environment a person is born into, and lifestyle.

Disruption of the gut flora can happen due to a range of common, everyday lifestyle choices such as eating a limited range of foods, alcohol consumption, taking medication, sedentary lifestyle, smoking and lack of sleep.

How do these lead to poor gut health?

- Food allows bacteria to grow by providing essential nutrients. Following a whole-food, varied diet including different fresh fruits and vegetables will supply a arrange of nutrients to your system leading to more varied gut flora.

- Alcohol can, in the case of red wine, promote healthy bacteria such as clostridium, though this is an exception and only works in small quantities. Generally, alcohol when consumed regularly reduces the level of healthy bacteria in the gut.

- We take antibiotics to get rid of harmful bacteria, however, a common side effect is change to the diversity of the gut flora by killing good bacteria, Bifidobacterial for example, and this imbalance can last for several years.

- Physically active people have been proven to have increased levels of bacteria such as Akkermansia that supports a healthy metabolism.

- Smokers have an increased risk of inflamed bowl and reduced gut flora, once smokers quit their range of healthy gut bacteria has been shown to increase.

- The gut follows a circadian-like cycle. Those who are staying up late, doing shift work, or even new parents disrupt the cycle when they do not get enough sleep, or have a disrupted sleep pattern. This can lead to an increase in bacteria associated with obesity.

Work

Occupational factors often have a negative influence on the digestive system. Most commonly occupational stress and tension, fatigue, issues with poor posture, especially when sitting for long periods of time, frequent changes in the pace of work, shift work, and irregular or poor eating habits (quantity, quality and timing of meals) (Nutrition for Athletes, 2020).

Those who sit at a desk all day commonly have slower digestive systems from inactivity and reduced blood flow. Those who work shifts are more prone to upset stomach, constipation and heart burn due to the digestive tract following the circadian cycle, it may be at a reduced level of activity during the night, and this may be the shift workers waking hours so when they are eating.

Though professional athletes do not of course have other work commitments and their schedule will be dedicated to training and resting at the optimum times, many athletes still have work and family commitments outside of the training demands. A prime example is marathon runner Uriga Buta who formally represented Norway in the 2012 Olympics while

working as a Janitor (. This can lead to an excessive demand on time and lead to unusual routines and training schedules. Everson (2009) suggests that the need for quality sleep is just as important as the need for food and drink in maintaining a healthy, functioning body. At the top of the list of solutions is monitoring fiber intake and ensuring both soluble and insoluble fibers are plentiful in the diet, reduce fat intake by choosing lean meats and add extra protein to the diet (*Eating for a Healthy*, 2018).

The Need for Dietary Fiber for Those on Low-Carbohydrate Diets

Insoluble dietary fiber, indigestible plant waste, is essential to supporting a healthy digestive system by reducing the risk of constipation, reducing cholesterol levels, and even reducing the risk of colon cancer. It is most commonly found in carbohydrate heavy foods (fruits, vegetables and wholegrains) which are generally consumed in lower amounts when following popular diets like ketogenic diet and Paleo and limiting carbohydrate intake due to diabetes (*Dietary Fiber*, 2021). It is likely that quality sources of fiber will be left out in order to avoid unwanted carbs (*Adding Fiber*, 2018).

As with all nutrients the demands of an athlete will vary from those of a regular person, the daily recommended amount of dietary fiber can be more than double that of a regular recommendation; athletes 20-35g compared to 10-15grams for others (Thomas, n.d.). When focusing a diet around high-fat foods such as eggs and full-fat dairy, whole-food fiber is missing, though it can be added by consuming foods such as avocado, flax seed and greens like bok choy (*Adding Fiber*, 2018). Again, the careful consideration of an athlete's daily calorie burn and macro, micro-nutrient needs and training schedule must be considered.

Fiber slows the emptying of the GI and so consuming fiber rich veggies like broccoli close to a workout can cause discomfort from gas and bloating (Wheller, 2020). It is common for endurance athletes to reduce fiber intake on the days before an event. Recent research done in Japan with college athletes found that fiber supplements such as Fiberpro were not, however, advisable alternatives to natural sources as they led to increased aggression and an increase in negative feelings among both male and female athletes (Sugiyama et al., 2017, p. 561).

In summary

This article has provided a basic introduction to macro and micronutrients, the digestive system and general details on the needs of athletes.

Overall, the need for personalized, adaptable and detailed planning is needed to meet an athlete's needs and ensure performance goals are met while maintaining a healthy body and mind.

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Chapter 17

EFFECT OF QUERCETIN IN HEPATOCELLULAR CARCINOMA

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1. Introduction

The liver is in charge of the entire body's metabolism. It's a matter of immunity. It is also the body's largest organ, with a large number of innate and congenital immune cells (Michalopoulos, 2020; Satriano et al. 2019; Ruf et al. 2021). Regulation of detoxification of toxins is involved in many important activities, such as balancing the intake of glucose, lipids, and amino acids, and converting them into other organic chemicals (Michalopoulos, 2020; Satriano et. al. 2019). The gut-liver axis exposes the liver to viral, fungal, and bacterial antigens. (Shin et al. 2021). Chronic inflammation linked to chronic viral hepatitis, high alcohol consumption, and hepatocyte damage, cause obesity or metabolic syndrome. Liver disorders such as alcoholic and non-alcoholic steatohepatitis, steatosis, nonalcoholic fatty liver disease (NAFLD), liver inflammation, and hepatic fibrosis develop as a result of hepatocyte damage. Hepatic chronic injury often causes liver cirrhosis and, in most cases, hepatocellular carcinoma (HCC) (Shin et al. 2021; Ruf et al. 2021; Fernández-Palanca et al. 2019). It plays a crucial role in the initiation, development, and progression of HCC. For the development of disease diagnosis and treatment, it is critical to understand the mechanisms that shape the unique tumor microenvironment of liver cancer and lead to tumor formation (Ruf et al. 2021).

1.1. Hepatocellular Carcinoma

It is estimated that the number of liver cancer patients will be over 1 million by 2030. There are several different types of liver cancer (Tseng et al. 2020). In adults, HCC is the most common type of mainly pathological liver cancer, which constitutes 90% of all cases. It is an important disease that causes more than 700,000 deaths per year worldwide (Li et al. 2021; Bresnahan et al. 2020; Tseng et al. 2020). It lacks effective therapeutic targets and it is difficult to find effective treatments with rapid proliferation (Li et al. 2021).

It is the world's fifth most common cancer type, and it is the second leading cause of cancer-related deaths (Llovet, et al. 2021; Pan et al. 2021). It is one of the most common diseases in the world. By 2030, it is expected that roughly 22 million new cancer diseases will be diagnosed, with 13 million cancer deaths per year (Sharifi et al. 2020). HCC is the primary malignancy in the liver and ranks second in cancer deaths in the world (Feng et al. 2021) Liver cirrhosis and non-alcoholic steatohepatitis (NASH) are on the rise among the causative factors of chronic liver disease. (McGlynn et al. 2021; Pinato et al. 2020; Bresnahan et al. 2020; Fujiwara et al. 2018).

In a study, it was stated that hepatitis B virus (HBV) constitutes 33% of liver cancer and death cases in the world. This is followed by the abuse of alcohol with a rate of 30%, the hepatitis C virus (HCV) with a rate of 21%, and it is explained that it constitutes other causes at 16%. In addition, it has been stated that the factors causing the disease vary significantly between countries and regions. (McGlynn et al. 2021; Singal et al. 2020; Bresnahan et al. 2020). The highest rates of HCC in the world are seen in Asia and Africa. (McGlynn et al. 2021).

Chemotherapy, targeted therapy, liver transplantation, surgical resection, radiofrequency ablation, trans-arterial chemoembolization, and immunotherapy are all options for treating HCC. The 5-year survival rate of HCC has been reported to be eighteen percent (Wu et al. 2021). In HCC, the patient is sensitive to only a few anticancer drugs. Most patients with intermediate or advanced HCC inapparent to respond effectively to anticancer drugs. The overall survival rate of patients with HCC is decreasing due to drug resistance. As a result, understanding the drug resistance mechanism of HCC, identifying drug-resistant points, and optimizing the treatment plan is critical for improving patient survival and saving lives in HCC patients (Wu et al. 2021).

The biggest challenge of cancer treatment is resistance to treatment due to the cancer cells' ability to escape apoptosis. Therefore, the development of new therapeutic agents is crucial to overcome treatment resistance (Li et al. 2021; Niazvand et al. 2019).

Chemoprevention is the case of using natural, biological or synthetic agents to prevent, inhibit, reverse or prevent invasion of premalignant cells in the initial stage of carcinogenesis. Interest in the field of chemo-prevention has greatly increased. With increasing interest, success is achieved in understanding cancer biology, determining molecular points and preventing certain types of cancer. Cancer chemoprevention is distinguished at the molecular level by altering multiple pathways that play a critical role in the three basic steps of carcinogenesis, namely initiation, progression, and progression step (Ranjan et al. 2019; Liao et al. 2015).

Prevention strategies are classified into three groups. Primary prevention focuses on preventing cancer-causing factors from entering the body, changing one's lifestyle, or eradicating the disease in its early through vaccination or environmental interventions tailored to the etiology. Early detection and chemoprevention of HCC formation or relapse in patients who have already been exposed to aetiological agents include secondary or tertiary prevention strategies. After radical HCC treatment, tertiary prevention aims to reduce widespread recurrence due to the spread of

residual tumor cells or de novo carcinogenesis in fibrotic / cirrhotic livers (Fujiwara et al. 2018). Despite advances in chemotherapy, survival rates for patients with advanced cancer remain low, indicating a critical need for new and more effective therapeutic strategies (Sak 2021). The use of medicinal plants against diseases has been increasing in recent years due to the side effects seen in the chemical drugs used in the treatment and the high cost of drugs.

1.2. Flavonoids

Bioflavonoids with various biological qualities like anticancer, antioxidant, anti-inflammatory, antibacterial, and antiviral activities are obtained from fruits and vegetables (Molani & Kheirouri 2021). There are more than 4000 bioflavonoid compounds in plants (Ponomarev et al. 2021).

Flavonoids are polyphenolic compounds contained in fresh fruits and vegetables, as well as some dietary supplements (Pang et al. 2021). By removing ligands from their binding sites, flavonoids can alter the pharmacokinetics of other drugs taken with them. For this reason, they may cause a strong or weak response, or they may have no effect on the ligand response at all (Wani et al. 2021).

They are polyphenolic compounds containing a wide range of born products, very rich in fruits and vegetables, are a group of secondary metabolites and known to have medicinal value from the past to the present (Mutha et al. 2021; Patil et al. 2021; Pang et al. 2021). Flavonoids are low molecular weight substances found in vascular plants (Ponomarev et al. 2021). Flavonoids are a class of compounds made up of a 15-carbon skeleton linked by flavan nuclei or pyran rings to two benzene rings. They are natural compounds made up of over 6000 well-defined molecules. (Patil et al. 2021).

Flavonoids, which are made up of polyphenols, can be commonly found in a variety of plants that are used for both medicine and food (Pang et al. 2021). Until today, more than 8,000 different flavonoids have been documented. The majority of these flavonoids can be found in the cells or surfaces of various plant tissue organs (Mutha et al. 2021).

Flavonoids stop cancer cells from multiplying by blocking the cell cycle or inducing apoptosis. In addition, flavonoids have a broad spectrum of action as protection against ultraviolet radiation, can protect molecules

and the intestinal epithelium from oxidative damage during digestion. It is explained that it has antihypertensive, antinecrosis, antioxidant, anti-inflammatory, antitumor, antiarrhythmic, hepatoprotective, antiviral effect, has an effect such as tumor angiogenesis, a decline of multidrug resistance in tumor cells. (Molani & Kheirouri 2021; Pang et al. 2021; Ponomarev et al. 2021). Flavonoids also affect many pathways linked to cancer development. It is also disclosed to have a cytotoxic or cytoprotective effect on tumor cells by affecting more than one target (Pang et al. 2021).

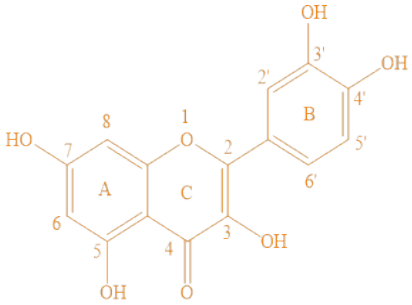

Figure 1. *Quercetin*

It is a polyphenolic flavonoid (Bagheri et al. 2021). Fruits (especially citrus fruits) and green leafy vegetables, as well as a variety of seeds, nuts, buckwheat, flowers, shells, broccoli, strawberries, red apple, olive oil, apple, red onion, onion, red grape, green tea, red wine, dark cherry, and one of the most abundant flavonoids in fruits like blueberries and cranberries (Bagheri et al. 2021; Adegoke et al. 2021). Their soluble properties have been investigated. It is indicated as highly soluble in lipids and alcohol, mildly soluble in hot water, and insoluble in cold water, according to its solubility. (Adegoke et al. 2021). One of the most widely utilized bioflavonoids for the treatment of inflammatory and metabolic disorders, it is pointed out to be. It has been described to interact with salivary proteins when ingested and forming soluble protein-quercetin binary clusters. (Shabbir et al. 2021).

Quercetin is the most important medicinal plant found in plants, such as fruits and vegetables, which are consumed by humans. It is widespread used in the prevention and treatment of a variety of diseases (Pan et al. 2021; Ibrahim et al. 2021).

1.3.1. Therapeutic Effect of Quercetin

Quercetin plays a role in scavenging the effects of reactive oxygen species (ROS) and has a higher antioxidant potential than other flavonoids. The presence of two pharmacophores, including the catechol group in

the B-ring and especially the hydroxyl group in the B-ring, is linked to quercetin's talent to act as a direct antioxidant (Bagheri et al. 2021; Ganesan et al. 2015; Zheng et al. 2017). In addition, it has been stated that neutralizes oxidative stress by inducing antioxidant enzymes after changing the Nrf2-ARE pathway (Costa et al. 2016).

Quercetin has traditionally been noted to have significant effects for the treatment of analgesic, antioxidant, antiviral, antiulcer, anti-hypercholesterolemic, anti-atherosclerotic, antispasmodic, antidiabetic, anti-inflammatory, antihypertensive, venoprotective, carcinogenesis, vasodilator functions, iron deficiency and many other diseases (Ghafouri-Fard et al. 2021; Bagheri et al. 2021; Wani et al. 2021; Almatroodi et al. 2021; David et al. 2016; Vásquez-Garzón et al. 2013).

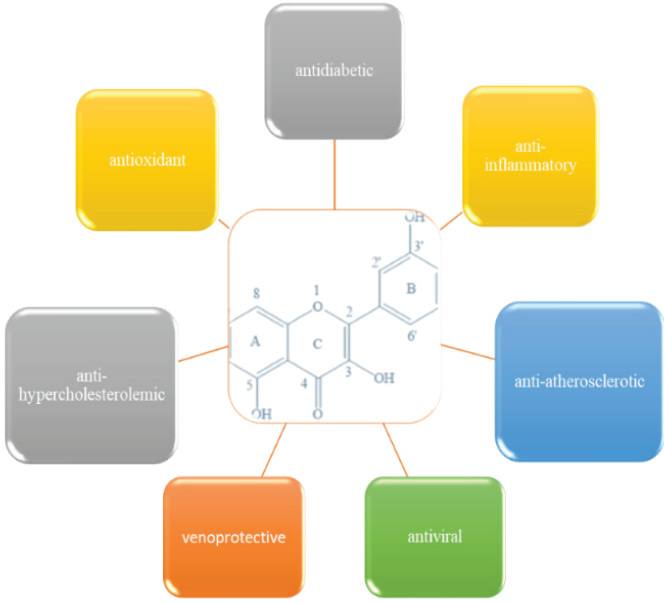

Figure 2. *Some therapeutic effects of Quercetin*

Quercetin has been found in more than 20 herbs recommended for treatment (Ghafouri-Fard et al. 2021). It is also used to cure diabetes, cataracts, cancer, obesity, and many neurological diseases (Bagheri et al. 2021; Dini et al. 2021; Amanzadeh et al. 2019).

1.3.2. Antioxidant Effect of Quercetin

The antioxidant talent of quercetin has been explained to protect low-density lipoproteins from free radical damage (de Whalley et al. 1990).

Quercetin is an antioxidant that protects against reactive nitrogen species, superoxide radicals, and peroxynitrite (de Lacerda Alexandre et

al. 2021; Boost et al. 2008). Some of Quercetin's antioxidant properties have been linked to its talent to chelate transition metal ions like Cu^{2+} and Fe^{2+}. (Sestili et al. 1998). Quercetin cleans superoxide anion radicals, blocks mitochondrial H_2O_2 formation, and also through a mechanism involving Fe^{2+} chelating activity, reduces lipid peroxidation (Devienne et al. 2007; Dorta et al. 2008).

Under control conditions, quercetin has no effect on endogenous antioxidants; however, in an isoproterenol-induced heart infarction model, quercetin pretreatment has prevented the decline of antioxidant enzymes (Punithavathi & Prince 2010; Liu et al. 2019).

1.3.3. Anticancer Effect of Quercetin

Quercetin has been explained to have anti-cancer properties, as well as the talent to induce intrinsic and extrinsic apoptotic pathways, cell cycle arrest, and autophagy (Wang et al. 2021). When compared to standard chemotherapeutic treatments, quercetin has been shown to be relatively safe and has no side effects even after a few months of oral administration (Wang et al. 2021; Ozarowski et al. 2018). However, the mechanisms underlying its anti-cancer effects have yet to be fully understood (Wang et al. 2021).

Quercetin is not harmful to healthy cells, but has been described to be toxic to cancer cells through a variety of mechanisms. This property makes quercetin a great candidate for anticancer treatments (Yin et al. 2021).

Along with its pro-apoptotic effect on tumor cells, quercetin has the ability to slow the progression of some human cancers also (Ghafouri-Fard et al. 2021; Granado-Serrano et al. 2008). Despite its high cytotoxic effects on cancer cells, quercetin has been shown to have little or no negative effects on normal cells because it does not affect normal cell proliferation. In studies conducted by creating in vitro and in vivo models to examine the therapeutic effect of quercetin, it has been explained that quercetin can prevent the initiation, development and formation of cancer, exhibit anti-carcinogenic and differentiation induction functionalities (Ghafouri-Fard et al. 2021; Yin et al. 2021). In studies, it has been evaluated the effect of in different types of cancer (Ghafouri-Fard et al. 2021).

When animal models of prostate cancer were studied in part of studies to examine the role of quercetin in prostate cancer, the majority of studies examined the effect of prostate cancer cells and explained that quercetin affects the activity of several signaling pathways, such as the Sonic Hedgehog signaling pathway (Ghafouri-Fard et al. 2021; Liu et al. 2019). When the effect of quercetin on cervical cancer is investigated, it

has been proven that it inhibits other pathways such as PI3K, MAPK, and WNT (Ghafouri-Fard et al. 2021; Kedhari Sundaram et al. 2019).

When its effect on the ovary was investigated, it was seen that it reduced cadmium-induced toxicity in the rat uterus and ovaries via antioxidant and anti-apoptotic effects and that it also protected ovarian granulosa cells from toxicity and apoptotic death in vitro studies (Nna et al. 2017; Capcarova et al.2015). Studies in mice have been shown to increase ovarian antioxidant capacity in ovarian granulosa cells during menopause and in vitro (Wang et al. 2018). Furthermore, quercetin has been demonstrated to inhibit carcinogenesis in colorectal cancer through different mechanisms that affect cell proliferation, reactive oxygen species production, and miR-21 expression (Ghafouri-Fard et al. 2021; Pratheeshkumar et al. 2017; Han et al. 2016). Furthermore, quercetin inhibits cell proliferation in normal breast epithelium without causing cytotoxicity in breast cancer cells (Ghafouri-Fard et al. 2021; Jeong et al. 2009). When its effect on lung cancer was examined, it was observed that it was effective in Akt and ERK phosphorylation in resistant lung cells, suppressed p-Akt and p-ERK, and improved paclitaxel cytotoxicity (Wang et al. 2021).

Quercetin Effect on HCC

HCC is a major disease that affects many people around the world. Different methods are used for the treatment of the disease. In the treatment process, both the costly treatment process and the side effects of the drugs affect the patient negatively. Therefore, it is important to develop new treatment methods. (Li et al. 2021; Sak 2021)

Medicinal herbs are used to treat a variety of ailments, and quercetin is an important bioflavonoid. It's used to treat a wide range of illnesses, including HCC. (Lee et al. 2015). Quercetin's positive effect on HCC has been demonstrated in many in vivo and in vitro experiments (Wu et al.2019). In studies, the effects of HepG2, Huh7, LM3 and SMMC-7721 on typical hepatocellular carcinoma cells and liver experimental animal models have been investigated. Besides, it has been explained that the SP1, p53, PI3K/PKC and MEK / ERK pathways have an effect on the regulation of expression and function (Wu et al. 2019; Bishayee et al. 2015; Lee et al. 2015; Muarya & Vinayak 2015; Ding et al. 2018).

Several studies have report quercetin to have antiproliferative and proapoptotic effects in HCC. In these studies, it is explained that quercetin has beneficial activities, antioxidant and anti-inflammatory properties and hepatoprotective effects (Zhang et al. 2021; Fernández-Palanca et al. 2019; Wu et al. 2019; Zou et al. 2018; Sudan & Rupasinghe 2015).

When quercetin's effect on HCC was investigated, it was seen that it inhibited the STAT3 mechanism in diffuse large B cell lymphoma cells (Almatroodi et al. 2021; Wu et al. 2019; Xiong et al. 2014)

It was found to prevent c-Met phosphorylation between human medulloblastoma cell lines, significantly preventing the invasion and migration induced by the hepatocyte growth factor in the cell line and the human liver cancer cell line HepG2. (Almatroodi et al. 2021; Labbe et al. 2009; Lee et al. 2006).

2. Conclusion

HCC is an important disease that is quite common worldwide and is the leading disease in the mortality rate. The factors causing the disease differ between societies and regions. The treatment process of the disease consists of different stages. As with all diseases, it is important to define early diagnosis and stage well.

Flavonoids have many important properties. Quercetin is also an important bioflavonoid and is frequently used for therapeutic purposes. In the study, the properties of quercetin, which is an important compound, and its effect on hepatocellular carcinoma were aimed to be explained.

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Chapter 18

WHO – 2017 CLASSIFICATION OF THYROID TUMORS AND CLINICOPATHOLOGIC SIGNIFICANCE OF THE REVISION

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Introduction

The World Health Organization (WHO) classification of thyroid tumors was published in the fourth edition of the WHO series in 2017 to provide the international standards for cancer diagnosis including diagnostic criteria, pathological features, and molecular alterations. The most significant changes in the 2017 WHO classification of thyroid tumors involve (1) molecular and genetic characterization of follicular-derived thyroid tumors; (2) a new classification for encapsulated well-differentiated follicular tumors, in particular, introduction of tumors of borderline malignancy or uncertain malignant potential; (3) changing the ICD-O (International Classification of Diseases for Oncology) behavior code of hyalinizing trabecular tumor to /1 (borderline or uncertain); (4) identification of new variants of papillary thyroid carcinoma (PTC); (5) subclassification of follicular thyroid carcinoma (FTC) into minimally invasive, encapsulated angioinvasive and widely invasive types; (6) reclassification of Hürthle cell adenoma/carcinoma and poorly differentiated thyroid carcinoma (PDTC) as distinct entities; and (7) changing the term carcinoma showing thymus-like differentiation to intrathyroid thymic carcinoma (Table 1.) (Lloyd et al. 2017, Bai et al. 2020).

In this review, we briefly introduce current histopathological classification of thyroid tumors, changes in the WHO-2017 classification of thyroid tumors, and update the contemporary diagnosis and classification of thyroid tumors since the revision.

In the fourth edition, a borderline tumor entity was incorporated to reduce the diagnostic discordance, and a nuclear score guide for *RAS* type PTC was provided. It was also aimed to reduce overdiagnosis and overtreatment of low-risk PTCs. The fourth edition further modified the definition of PTC and stated that "PTC is an epithelial malignant tumor with evidence of follicular cell differentiation and a set of distinctive nuclear features. PTC is usually invasive. Papillae, invasion, or cytopathological features of PTC are required." These changes were also intended to reduce overdiagnosis and overtreatment of low-risk PTCs, and the majority of encapsulated PTCs without clear-cut invasion were downgraded to the borderline tumor category, including well-differentiated tumor of uncertain malignancy potential (WDT-UMP) and noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP), because PTCs were still heterogeneous tumors genetically, and the nuclear feature alone was not successfully correlated with genetic alterations (*RET*/*PTC* rearrangements, *BRAF*V600E mutation, and *RAS* mutations) and accurate identification of the true biological nature of the tumors (Bai et al. 2020).

In the fourth edition WHO classification, FTC was classified into three prognostic groups: (1) minimally invasive (capsular invasive only) FTC; (2) encapsulated angioinvasive FTC; and (3) widely invasive FTC. Furthermore, in the follicular adenoma (FA)/FTC lineage, significant numbers of low-risk FTC (minimally invasive FTC) were downgraded to the borderline tumor category (follicular tumor of uncertain malignancy potential [FT-UMP]) using stricter criteria of capsular and vascular invasions. It was also intended to reduce overtreatment for low-risk FTCs.

Table 1. *WHO 2017 Revisions to the Classification and Nomenclature of Thyroid Tumors.*

New entities and ICD-O codes added		ICD-O behavior codes changed
Follicular tumor of uncertain malignancy potential (FT-UMP),		Hyalinizing trabecular tumor (/0 => /1)
Well-differentiated tumor of uncertain malignancy potential (WDT-UMP),		Ectopic thymoma (/1 => /3)
Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP),		In ICD-O, behavior codes /0 and /1 mean benign and borderline / tumors of uncertain malignancy potential, respectively
Encapsulated variant of PTC, Encapsulated angioinvasive FTC,		ICD-O: International Classification of Diseases for Oncology; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma.
Hürthle cell adenoma,		
Hürthle cell carcinoma		
New designation of ICD-O codes for		New terms changed =>
Follicular variant of PTC,		Follicular adenoma, oncocytic type => Hűrthle cell adenoma
Papillary microcarcinoma,		Follicular carcinoma, oncocytic type => Hűrthle cell carcinoma
Columnar cell variant of PTC,		Undifferentiated (anaplastic) carcinoma => Anaplastic thyroid carcinoma
Oncocytic variant of PTC,		Mixed medullary and follicular cell carcinoma => Mixed medullary and follicular thyroid carcinoma
Minimally invasive FTC,		Carcinoma showing thymus-like differentiation => Intrathyroid thymic carcinoma
Poorly differentiated thyroid carcinoma		

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In the ICD-O codes, the morphology code records the histopathological type of the neoplasm and its biological activity in terms of how it behaves. Behavior codes /0, /1, /2, and /3 mean benign neoplasms, neoplasms of uncertain behavior, carcinoma in-situ, and malignant neoplasm, respectively. The behavior code /2 has not been assigned to thyroid tumors.

Follicular Adenoma and Borderline (UMP: uncertain malignancy potential) Tumors

The 2017 edition of the WHO book on Classification of Thyroid Tumors (Lloyd et al. 2017) includes a new section entitled 'Other encapsulated follicular-patterned thyroid tumors' in which, together with the two types of 'UMPs' ('Follicular tumor of Uncertain Malignancy Potential' and 'Well differentiated tumor of Uncertain Malignant Potential'), the newly created NIFTP (Non-invasive follicular thyroid neoplasm with papillary-like nuclear features) is identified and described in detail (Cameselle-Teijeiro et al. 2017, Lloyd et al. 2017, Amendoeira et al, 2018).

The introduction of borderline tumors (hyalinizing trabecular tumor, NIFTP, and tumors of uncertain malignancy potential) to thyroid tumor classification is a critical change in thyroid pathology practice (Carney et al. 2008, Kakudo et al. 2012, Kakudo et al. 2018). With this breakthrough change, thyroid tumors are classified into three risk groups by the probability of recurrence/metastasis: negligible risk (<0.1%) in benign tumors, very low risk (<1%) in borderline tumors, and high risk in malignant tumors. FA is a benign, encapsulated, noninvasive neoplasm showing evidence of thyroid follicular cell differentiation, without nuclear features of PTC. A new phrase, "without nuclear features of PTC," was added to the previous definition by the 2004 WHO classification of thyroid tumors, because noninvasive encapsulated follicular pattern tumors with PTC-like nuclear features are excluded from FA and are classified in NIFTP or WDT-UMP in the new definition (Lloyd et al. 2017).

The concept and the designation NIFTP that encompasses former E/NI-FVPTC and WDT, UMP diagnosed due to equivocal PTC-like nuclear features, intend to constitute a way of solving the clinicopathologic problems through the utilization of terms ('non-invasive' and 'tumor' instead of 'carcinoma') that will induce a more conservative treatment. This option will reduce the iatrogeny, psychosocial burden and high economical costs for patients and medical care. Despite deleting the word 'carcinoma' from its name, NIFTP is not a benign tumor either and is a neoplasm with 'very low malignant potential', since rare cases fulfilling its criteria may give rise to metastasis (Cho et al. 2017, Parente et al. 2017, Amendoeira et al. 2018).

Strict inclusion and exclusion criteria are necessary to support the diagnosis of NIFTP (Nikiforov et al. 2016). Inclusion criteria are the following: follicular pattern, encapsulation/good circumscription and nuclear grade equal or above 2 (a nuclear grade score 0–3 was created to define minimal nuclear 'atypia' necessary to render a diagnosis of NIFTP instead of a diagnosis of follicular adenoma/adenomatous nodule). Assuming the aforementioned entry criteria are present, the final diagnosis of NIFTP may be rendered if all of the following exclusion features are absent: vascular or capsular invasion, more than 1% papillae, presence of psamomma bodies, more than 30% solid-trabecular architecture, more than 3 mitoses/10 HPF and presence of tumor necrosis (Nikiforov et al. 2016).

Several studies reported significant interobserver variation in PTC-type nuclear features and PTC-like nuclear features in the diagnosis of encapsulated follicular pattern thyroid tumors which also should affect FA diagnosis (Thompson et al. 2018, Hirokawa et al. 2002, Liu et al. 2019, Lloyd et al. 2004).

The two types of nuclear features are called either (1) PTC-type nuclear features that have fully developed PTC-type nuclear changes (often observed in *BRAF* V600E mutated PTCs) and (2) PTC-like nuclear features (delicate nuclear changes) defined by Nikiforov et al. using the nuclear score guide that were often seen in *RAS* mutated PTCs and NIFTP.

The 2017 edition of the WHO book on classification of thyroid tumors includes a new section entitled 'Other encapsulated follicular-patterned thyroid tumours', in which the newly created NIFTP (non-invasive follicular thyroid neoplasm with papillary-like nuclear features) is identified and described in detail. NIFTP is not a benign tumor but is a neoplasm with 'very low malignant potential'. The main goal of the introduction of NIFTP category is to prevent overdiagnosis and overtreatment. Sampling constraints, especially when dealing with heterogeneous and/or large nodules, and difficulties in the invasiveness evaluation, are the major weaknesses of the histological characterization of NIFTP. At the cytological level, NIFTP can be separated from classic papillary carcinoma (cPTC) but not from encapsulated, invasive follicular variant PTC. The impact of NIFTP individualization for cytopathology is the drop of rates of malignancy for each Bethesda category in general and for indeterminate categories in particular. The biggest impact will be seen in institutions with a high frequency of FVPTC. The introduction of NIFTP has changed the utility of predictive values of molecular tests because RAS mutations and PAX8-PPARg rearrangements are frequently detected in NIFTP. This turns less promising the application of mutation detection panels as indicators of malignancy and will probably contribute

to switch to a rule-out approach of molecular testing (Amendoeira et al, 2018).

The majority of NIFTP cases are classified in the Bethesda 'Indeterminate' categories– atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular neoplasm/ suspicious for follicular neoplasm (FN/SFN) or even suspicious for malignancy (SM) (Strickland et al. 2015, Faquin et al. 2016, Maletta et al. 2016). Taking into consideration the morphological and molecular heterogeneity of NIFTP, it is not surprising the spreading of cytopathological diagnosis of NIFTP through different Bethesda categories, depending on the assessment of nuclear atypia (Amendoeira et al. 2018).

Most of the NIFTP diagnostic criteria are exclusion criteria, such as the percentage of papillary structures and of trabecular/solid areas that do not help to define an entity from a pathogenic or molecular standpoint. The same holds true regarding the inclusion in the NIFTP group of cases in which nuclei do not display full-blown PTC features (score 2) or have nuclei displaying score 3 features in part of a tumor that displays mainly follicular cell nuclei (Nikiforov et al. 2016). This cellular/nuclear heterogeneity probably justifies the heterogeneity of the molecular profile of NIFTP (Amendoeira et al. 2018).

The study by Cho et al evaluated the impact of the threshold of acceptance of papillary architecture to diagnose NIFTP and found significant differences regarding prevalence of BRAFV600E mutations when comparing 'NIFTP with no papillae at all' vs 'NIFTP with papillae' (less than 1%) (Cho et al. 2017). No mutations were present in cases with no papillae formation, whereas the BRAFV600E mutation was detected in some cases of the other group. Although the morphologic demonstration of vascular invasion is a much better predictor of increased aggressiveness than the presence of BRAFV600E mutation per se (Eloy et al. 2011), these findings pointed to the need to refine diagnostic criteria in order to try to obtain a more homogeneous molecular profile in NIFTP (Nikiforov et al. 2018).

Ohba et al. recently proposed a new borderline thyroid tumor with a papillary growth pattern and named it noninvasive encapsulated papillary RAS-like thyroid tumor (NEPRAS). Subsequent studies reported more cases of NEPRAS (Ohba et al. 2019, Jung et al. 2020, Rosario 2020). NEPRAS is an encapsulated papillary patterned thyroid tumor without capsular or vascular invasion, mild nuclear enlargement and nuclear membrane irregularity which correspond to a nuclear score of 2.

Noninvasive encapsulated papillary patterned thyroid tumors with papillary thyroid carcinoma (PTC)-like nuclear features were not defined

in the fourth edition World Health Organization classification of tumors of thyroid (Lloyd et al. 2017). Ohba et al. proposed to name it noninvasive encapsulated papillary RAS-like thyroid tumor (NEPRAS) (Ohba et al. 2019).

Hyalinizing Trabecular Tumor

A hyalinizing trabecular tumor is a rare thyroid neoplasm of follicular cell origin with very low malignant potential. The tumor was moved from a benign group of "thyroid adenoma and related tumors" in the 2004 WHO classification to a separate category, where it is classified as a neoplasm of uncertain behavior with an ICD-O behavior code of /1 in the 2017 WHO classification (Lloyd et al. 2017).

Because hyalinizing trabecular tumors have PTC-type nuclear features, including intranuclear grooves and pseudoinclusions, they can be challenging to diagnose in fine-needle aspiration cytopathology. More than half of the tumors were diagnosed as PTC or suspicious for PTC (Nikiforova et al. 2020).

Recent studies reported the *PAX8*/*GLIS* fusions to be a genetic hallmark of hyalinizing trabecular tumor (Nikiforova et al. 2020, Marchio et al. 2019). *PAX8*/*GLIS* fusions have not been found in other types of thyroid tumors to date (Nikiforova et al. 2019, Nikiforova et al. 2020). Because all hyalinizing trabecular tumors with fully developed histopathologic features of the tumor had *PAX8*/*GLIS* fusions and benign clinical behavior, Nikiforova et al. in 2019 suggested that the diagnostic term for these tumors should be "*GLIS*-rearranged hyalinizing trabecular adenoma" changing the current term of "tumor" to "adenoma."

Papillary Thyroid Carcinoma (PTC)

In accordance to the construction of a new borderline tumor category. so many PTC variants are recognized, including classic, microcarcinoma (≤1 cm in diameter), encapsulated, follicular, diffuse sclerosing, tall cell, columnar cell, cribriform-morular, hobnail, PTC with fibromatosis/fasciitis-like stroma, solid/trabecular, oncocytic, spindle cell, clear cell, and Warthin-like variants (Table 2) (Lloyd et al. 2017, Bai et al. 2020).

Table 2. *Histopathologic Diagnostic Criteria for the PTC Variants (Bai et al. 2020).*

PTC Variant	Percent of variant tumor cells	Histopathologic diagnostic criteria
Follicular variant	100%	No true papillae, follicular pattern, PTC nuclear features
Tall cell variant	≥30%	Elongated follicles, closely packed papillae, tall cells 2-3 times taller than wide, abundant eosinophilic cytoplasm, sharply delineated cell borders
Columnar cell variant	≥30%	More elongated cells than tall cell variant, pseudostratified hyperchromatic nuclei, lack conventional PTC nuclei, subnuclear cytoplasmic vacuoles
Hobnail variant	≥30%	Loss of cellular cohesion, micropapillary, apically located pleomorphic nuclei, prominent nucleoli, mitosis
Solid variant	nearly 100%	Solid, trabecular, or insular pattern, PTC nuclear features, absence of high mitosis and/or necrosis
Diffuse sclerosing variant	NA	Dense sclerosis, numerous psammoma bodies, lymphatic invasion, chronic lymphocytic thyroiditis, squamous metaplasia
Other variants	>50%	

PTC, papillary thyroid carcinoma; NA, not available.

Among PTC variants, tall cell, columnar cells, and hobnail variants are of undoubted clinical significance, since they are aggressive variants associated with negative clinicopathological features and worse prognosis than for classic and encapsulated PTC (Lloyd et al. 2017, Nath and Erickson 2018, Ho et al. 2020). In the 2015 ATA (American Thyroid Association) risk stratification system, patients with these three aggressive variants are classified within the intermediate risk group Haugen et al. 2016).

Although solid and diffuse sclerosing variants are regarded as aggressive variants of PTC by some researchers, their biological behaviors are still controversial (Lloyd et al. 2017, Nath and Erickson 2018, Ho et al. 2020). An increase in the incidence of aggressive PTCs was observed at a rate higher than that seen in well-differentiated PTCs or anaplastic thyroid carcinomas (ATCs) in the past two decades. There is increasing evidence that when aggressive variants of PTCs present as low stage and without invasive features (including extrathyroid invasion, tumor thrombi, and lymph node metastasis), they might seem rather indolent (Song et al. 2018, Limberg et al. 2019).

Tall cell variant of PTC

The definition of the tall cell variant was revised in the current WHO classification, because at least 30% of tumor cells are two to three times as tall as they are wide.

There is increasing evidence that PTCs with as little as 10% tall cell change have a worse prognosis than do those without tall cells, and that PTCs with focal tall cell change (≥10%) should be reported and handled beyond the low-risk classification (Ganly et al. 2014, Beninato et al. 2013, Dettmer et al. 2015, Vuong et al. 2018, Bongers et al. 2019, Oh et al. 2014). The diagnosis of the tall cell variant should not be missed, in that patients with this variant are more refractory to radioactive iodine ablation and have a worse prognosis than do those with classic PTC (Rivera et al. 2008, Silver et al. 2011, Shi et al. 2016). *BRAF* V600E and *TERT* promotor mutations are the most common genetic alterations for this variant (Dettmer et al. 2015, Villar-Taibo et al, 2017).

Columnar cell variant of PTC

The columnar cell variant consists of columnar cells with marked pseudostratified hyperchromatic nuclei and absence of conventional PTC nuclear features (Lloyd et al. 2017, Jung 2020). CDX2, a gut-specific nuclear transcription factor, the expression of which is a putative feature of intestinal-type differentiation, is positive in 10% to 55% of this variant (Enriquez et al. 2012, Sujoy et al. 2013), which may behave indolently or aggressively depending on tumor encapsulation and tumor size (Yunta et al. 1999, Huang et al. 2005). Columnar cell variants of circumscribed or intrathyroidal types have an excellent prognosis, whereas invasive tumors with extrathyroidal extension have a less favorable prognosis (Chen et al. 2011).

Columnar cell variant of PTC is composed of papillary and glandular architecture lined by columnar cells showing nuclear stratification and lacking characteristic nuclear features of PTC. Columnar cells resemble secretory-type endometrium having supranuclear cytoplasmic vacuoles (Bai et al. 2020).

Hobnail variant of PTC

The hobnail variant is a novel entity in the 2017 WHO classification of tumors of thyroid, defined by >30% of tumor cells with hobnail features (Lloyd et al. 2017). It is rare and accounts for <3% of all PTC cases (Ieni et al. 2016). Hobnail features in PTC were firstly described by Kakudo et al. in 2004 as loss of cellular polarity and cohesiveness, with the main growth pattern of micropapillary and papillary structure, which were correlated

with a higher risk of recurrence. This feature represented an aggressive growth with poor prognosis, in that most cases had lymph node/distant metastases and local recurrence, An epithelial-mesenchymal transition was suggested as a possible mechanism of metastasis for this variant (Bai et al. 2009, Liu et al. 2011, Ambrosi et al. 2017). *BRAF* V600E mutation is the commonest genetic alteration for this variant, accounting for 50% to 94% cases, followed by *TP53* mutation (Lee et al. 2015, Teng et al. 2017, Lubitz et al. 2014).

The hobnail variant must be differentiated from classic PTCs showing hobnail-like morphology, which is associated with papillae with hyalinized and edematous fibrovascular core (Wong et al. 2020). The hobnail-like morphology in classic PTC with cystic change is not a true hobnail variant but degenerative changes (Bai et al. 2020).

Solid variant of PTC

The solid variant should be identified when most of the tumor consists of solid / trabecular / insular components. A subset of solid variant PTCs, which lack conventional PTC nuclear features and present high mitotic figures and/or necrosis, fulfill the diagnostic criteria for poorly differentiated thyroid carcinoma (PDTC) and therefore are currently classified as PDTC (Lloyd et al. 2017, Volante et al. 2007).

This variant is strongly associated with ionizing radiation and is more common in young patients and children (Collini et al. 2006, LiVolsi et al. 2011, Nikiforov et al. 2001, Tronko 1999). Radiation-associated pediatric cases frequently show *RET*/*PTC3* rearrangement and sporadic cases show *RET*/*PTC1*/*3* and *ETV6*/*NTRK3* fusions (Nikiforov et al. 2001, Ohashi 2020). *BRAF* V600E mutations are less commonly found than are classical PTC.

Some earlier studies reported poorer prognosis in patients with solid variant PTC than in those with classical PTC, subsequent studies revealed no difference in prognosis between the variants (Nikiforov et al. 2001, Ohashi 2020, Ohashi et al. 2017).

Diffuse sclerosing variant of PTC

The diffuse sclerosing variant, which usually presents with rapid and diffuse enlargement of the thyroid gland, clinically may be mistaken for autoimmune thyroiditis. It is more commonly seen in young women and is characterized by marked stromal fibrosis, dense lymphocytic infiltration, numerous psammoma bodies, and foci of squamous metaplasia, as well as frequent lymphovascular invasion (Lloyd et al. 2017, Caplan et al. 1997). *RET*/*PTC* rearrangements represented the genetic initiation event

for this variant (Joung et al. 2016, Sheu et al. 2007, Pillai et al. 2015). This variant is associated with extrathyroidal extension, cervical lymph node and distant metastasis, and shorter disease-free survival; however, the mortality rate is comparable to that of the classical variant (Thompson et al. 2005, Koo et al. 2009, Fukushima et al. 2009, Malandrino et al. 2016). The excellent long-term survival despite a higher recurrence rate may result from the favorable effect of a younger patient age.

Papillary microcarcinoma (mPTC)

Papillary microcarcinoma of the thyroid (mPTC) is defined by the WHO as a PTC ≤10 mm in diameter and it is currently a topic of intense confusion among the thyroidologists due to its "epidemic" rise. Although these tumors follow mostly an indolent clinical course and carry an excellent prognosis, it is well known that a small subset may display a potentially aggressive behavior. On the other hand, we still lack an accurate way of predicting those which will cause significant disease. In an attempt to address this problem, a number of clinicopathologic features have been studied as poor prognostic markers and their association with known genetic alterations in thyroid cancer has been evaluated in mPTC (Rodrigues et al. 2017).

Follicular Thyroid Carcinoma (FTC)

In the 2017 WHO classification, minimally invasive FTC is further classified as minimally invasive (capsular invasion only) and encapsulated angioinvasive (with or without capsular invasion). There is no revision in the diagnostic criteria for widely invasive FTC, which extensively invades the thyroid gland and extra-thyroidal soft tissue and often shows extensive vascular invasion. However, extensive angioinvasion alone does not categorize the FTC as being widely invasive FTC (Bai et al. 2020).

The extent of vascular invasion in FTC is prognostically relevant (Lloyd et al. 2017, Kim et al. 2014, Xu and Ghossein 2015, O'Neill et al. 2011). In encapsulated angioinvasive FTC, tumors with limited vascular invasion (<4 vessels) have a better outcome than do those with extensive vascular invasion (≥4 vessels) (Xu and Ghossein 2015). However, extensive vascular invasion did not affect the survival of patients with widely invasive FTC independently (Kim et al. 2014). The *TERT* promoter mutation was seen in FTC, but not in atypical FA or FT-UMP, and the *TERT* promoter mutation was significantly higher in patients with metastatic FTC than in patients with non-metastatic FTC (Cracolici et al. 2020).

The 2017 WHO classification defines three histological subtypes of follicular thyroid carcinoma: minimally invasive (with excellent prognosis), encapsulated angioinvasive, and widely invasive type (with higher risk of recurrence and metastatic spread). The fact that definite characterization of follicular neoplasms is predominantly a postoperative histological diagnosis (core criteria: capsular, vascular and adjacent tissue invasion) translates into the challenge for the thyroid surgeon to plan preoperatively for presence of malignancy and, if required, to adapt the surgical strategy according to intraoperative (frozen section) or postoperative histological findings. Until improved tools for pre-/intraoperative diagnosis are available, the malignant potential of a follicular thyroid lesion can be assessed by stratifying the patient according to clinical risk factors (presence of metastases, advanced patient age, tumor size). A stepwise, escalating surgical approach with restricted primary resection (hemithyroidectomy) and completion surgery based on the definite histopathology is another option to solve this dilemma. The currently recommended surgical treatment strategies for FTCs as published by ATA, BTA, CAEK and ESES are discussed. There is consensus that prophylactic lymphadenectomy is not required for FTCs and that hemithyroidectomy is sufficient in low-risk FTCs (capsular invasion only) whereas thyroidectomy with postoperative radioiodine therapy is indicated in high-risk FTCs (angioinvasion; widely invasive FTC) (Staubitz et al. 2019).

Hürthle (oncocytic) Cell Tumors

Hürthle cell tumors, should be diagnosed if the majority of the tumor (>75%) consists of oncocytic cells (Lloyd et al. 2017, Dettmer et al. 2015). Hürthle cell adenomas and carcinomas had been regarded as variants of FA and FTC, respectively, but they were nominated as a distinct entity in the 2017 WHO classification, since they have biological behaviors and molecular profiles different from those of follicular tumors (Maximo and Sobrinho-Simoes 2000, Bishop et al. 2012, Haq et al. 2005, Gasparre et al. 2007, Maximo et al. 2002). Hürthle cell carcinomas are different from conventional FTCs, in that Hürthle cell carcinomas occur in older patients, are more common in men, can develop cervical lymph node metastasis, and have larger tumors, higher-stage disease, and worse prognosis, and are resistant to radioactive-iodine treatment (Chindris et al. 2015). As with conventional FTC, the prognosis of Hürthle cell carcinoma is depended on the extent of vascular invasion (Xu and Ghossein 2015).

Integrated genomic analysis revealed that, besides recurrent mitochondrial mutations, whole-chromosomal duplications of chromosomes 5 and 7 and widespread loss of heterozygosity because of

haploidization and copy-number-neutral uniparental disomy were unique chromosomal landscapes for this special tumor (Ganly et al. 2018). *EZH1* mutation was found in a small subset of Hürthle cell adenoma and minimally invasive Hürthle cell carcinoma, but not in widely invasive Hürthle cell carcinoma (Jung et al. 2018).

Poorly Differentiated Thyroid Carcinoma (PDTC)

PDTC is a follicular cell neoplasm that is morphologically and behaviorally intermediate between well-differentiated thyroid carcinoma (WDTC) and ATC. Different definitions of PDTC have been reported. The most well-known ones are Sakamoto-type PDTC, Memorial Sloan Kettering Cancer Center (MSKCC) criteria, and the Turin proposal (Volante et al. 2007, Sakamoto et al. 1983, Hiltzik et al. 2006). Sakamoto et al. focused on the tumor growth pattern (solid, trabecular, and/or scirrhous) in 1983. The MSKCC criteria defined PDTC based on the tumor necrosis and/or mitotic figures (≥5/10 high-power fields) in 2006 (Hiltzik et al. 2006). In 2007, the Turin proposal covered the definitions by Sakamoto et al. and MSKCC criteria, and made a revision (Volante et al. 2007, Sakamoto et al. 1983, Hiltzik et al. 2006).

The 2017 WHO classification adopted the Turin consensus proposal as the diagnostic criteria for PDTC, including (1) diagnosis of carcinoma of follicular cell origin; (2) solid/ trabecular/ insular growth pattern; (3) absence of classical nuclear features of PTC; and (4) at least one of the following three features: convoluted nuclei, ≥3 mitoses /10 HPFs (high-power fields), or tumor necrosis (Lloyd et al. 2017, Volante et al. 2007). The convoluted nuclei refer to raisin-like hyperchromatic nuclei with irregular membranes.

Validation studies confirmed that the Turin consensus criteria could reliably select PDTC cases, and they reflected the patient prognosis more accurately than that in the 2004 WHO classification (Akaishi et al. 2019, Asioli et al. 2010). Although this algorithm was not initially used for Hürthle (oncocytic) cell carcinoma, it was proved to be practical and is now accepted as being useful for identifying oncocytic PDTC (Lloyd et al. 2017, Bai et al. 2015).

The Ki-67 index is a helpful marker used adjunctively in differentiating PDTC from WDTC and ATC, since it is usually 10% to 30% in PDTC, but <10% in WDTC and >30% in ATC (Ziad et al. 2008, Kakudo et al. 2015, Deeken-Draisey et al. 2018). Kakudo et al. also proposed a prognostic classification of thyroid tumors into benign, borderline, and malignant tumors by the Ki-67 labelling index. They proposed “high-risk thyroid carcinoma of follicular cell origin” defined by the Ki-67 index of 10-30%

to cover all histological types of high-risk carcinomas, listed in the WHO classification as one of the synonyms and related terms of PDTC.

PDTC could be pure or could coexist with PTC or other types of tumors. In the latter case, the percentage of PDTC should be reported. As little as 10% of PDTC in a background of well-differentiated tumors could be associated with aggressive features and unfavorable prognosis (Dettmer et al. 2011). The extent of invasion was an important parameter that affected clinical outcomes for patients with PDTC, and patients with encapsulated PDTC with capsular invasion only or focal vascular invasion (fewer than four foci) had an excellent outcome (Wong et al. 2019).

In accordance with its morphology and biological behavior, PDTC has genetic alterations embracing the alterations in PTC, FTC, and ATC: *BRAF* V600E, *RAS*, *TERT* promoter, *TP53* mutations, and other high-risk gene mutations (Ibrahimpasic et al. 2019). The prevalence of the *TERT* promoter mutation was significantly higher in tumors harboring aggressive histopathological features (including PDTC, tall cell variant of PTC, and widely invasive FTC) than in non-aggressive thyroid carcinomas, and *TERT* promoter mutations were associated with poor outcomes (Bournaud et al. 2019).

Well-Differentiated Thyroid Carcinoma with (WDTC) high grade features

Typically, mitoses are found to be <3 / 10 HPFs in the WDTC. The presence of high mitotic rates and/or necrosis raises the possibility of PDTC. However, according to the WHO classification, the high-grade features alone do not categorize the tumor as PDTC. The same tumors can be classified as PDTC by the MSKCC criteria if tumor necrosis and/or mitoses of ≥5 per 10 high-power fields are found in a follicular-derived carcinoma regardless of growth pattern (Hiltzik et al. 2006, Xu and Ghossein 2020).

Anaplastic Thyroid Carcinoma (ATC)

ATC is a highly aggressive malignancy that usually presents as advanced disease for which even radical surgery could not be possible. ATC can develop through dedifferentiation of a differentiated thyroid carcinoma or directly from follicular cells. Favorable patient survival was reported in cases with small foci of ATCs found incidentally within WDTCs after thyroid surgery (Keutgen et al. 2015). Therefore, the percentage of ATC components must be noted in the pathology report.

Histopathologically, ATC can consist of one or in any combination of three patterns: sarcomatoid (composed of spindle cells), giant cell

(composed of multinucleated giant cells), and epithelial (composed of squamoid or squamous tumor cells). All forms of ATC are characterized by dense inflammatory infiltrate, tumor-infiltrating macrophages, necrosis, and high mitosis. The rare histologic variants of ATC include paucicellular, rhabdoid, and small cell variants.

The diagnosis of ATC is challenging, since it overlaps morphologically with other malignancies showing anaplastic features, and the diagnoses should be made only from biopsy materials in most circumstances. The rational use of thyroid-specific markers as well as other tissue-specific markers is helpful in differentiating ATC from its mimics (Kuhn et al. 2019, Talbott and Wakely 2015). PAX8 is reported to be a sensitive marker for detecting cells of thyroid origin other than TTF1 and thyroglobulin (Kuhn et al. 2019, Bishop et al. 2011). CD10 is diffusely and strongly expressed in ATC but absent or focally in WDTCs (Nakazawa et al. 2018, Oh et al. 2020).

The *TERT* promoter mutation was most prevalent (75%), followed by *TP53* (63%), *BRAF* (45%), *RAS* (24%), *PIK3CA* (18%), *E1F1AX* (14%), and *PTEN* mutations (14%). Concomitant *TERT* promoter and *BRAF*/*RAS* mutations were associated with worse outcomes than were mutation in only one of the genes (Xu et al. 2020).

Medullary Thyroid Carcinoma (MTC)

Medullary thyroid carcinoma (MTC) accounts for only 0.5-3% of all malignant tumors, but is responsible for more deaths every year than all the other endocrine malignancies taken together. Approximately 75-80% of MTCs occur sporadically, while the inherited forms of MTC are responsible for the remaining cases. The heritable MTCs result from germline mutation in the rearranged during transfection (RET) proto-oncogene and is included into the multiple endocrine neoplasia 2 (MEN2), being associated with other endocrine abnormalities and clinical features. MTC is a neuroendocrine tumor that releases a wide range of secretory products that are responsible for a variety of symptoms, making it difficult to be diagnosed. For this reason, the pathological analysis is mandatory to ensure that the correct diagnosis is made (Buzdugă et al, 2019). The new approach to this type of cancer is described in the current WHO classification of thyroid tumors (2017) and the reassessment of MTC tumor category in the contemporary AJCC⁄TNM (American Joint Committee on Cancer⁄Tumor, Node, Metastasis) Staging (2017).

Thyroid Tumors Showing Thymus-like Differentiation

Thymic and third pharyngeal pouch remnants entrapped in the thyroid gland can give rise to ectopic thymoma, spindle epithelial tumor with thymus-like differentiation, and intrathyroid thymic carcinoma. The current 2017 WHO classification changed the term "carcinoma showing thymus-like differentiation" in the 2004 WHO classification to "intrathyroid thymic carcinoma" (Lloyd et al. 2017).

Intrathyroid thymic carcinomas share the immunohistochemical profile of primary thymoma that is positive for cytokeratin, CD5, KIT (also known as c-Kit and CD117), and p63, and negative for thyroglobulin and TTF1. Three histologic subtypes of this tumor include the squamous cell carcinoma type, lymphoepithelioma or basaloid type, and neuroendocrine carcinoma type (Bai et al. 2020).

NTRK-reerranged Thyroid Tumors

Although this new entity has not been listed in the WHO classification of tumors of thyroid, cancer-type classification based on genetic alterations is clinically relevant, given that tyrosine kinase inhibitors targeting somatic mutations and fusions have been approved for the management of cancer patients (Cocco et al. 2018).

Conclusion

The 2017 WHO classification categorized thyroid neoplasms into benign, borderline (uncertain malignancy potential), and malignant tumors. Certain types of thyroid cancers were reclassified as borderline tumors. Histopathologic variants of PTC and FTC were redefined to better stratify prognosis and management of patients.

Molecular-based classification for thyroid neoplasm allows diagnosis and prognostic risk stratification of thyroid cancer and may increase the accessibility of targeted treatments. Selection for surgery will go on being determined by a combined detection of clinical, cytopathological and radiologic suspicious features.

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