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EDITORS

PROF. DR. ZELİHA SELAMOĞLU

PROF. DR. HASAN AKGÜL

PROF. DR. ENGİN ŞAHNA

SERÜVEN YAYINEVİ

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Telefon / Phone: 05437675765
web: www.seruvenyayinevi.com
e-mail: seruvenyayinevi@gmail.com

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CONTENTS

CHAPTER 1

CURRENT APPROACHES TO SPECIES AND GENDER DETERMINATION OF MEATS.

Ali ARSLAN[1], Mehmet Emin AYDEMİR[2]

1 Prof. Dr. , Fırat University Faculty of Veterinary Medicine Department of Food Hygiene and Technology, ORCID:0000-0002-3011-5592
2 Dr., Harran University Faculty of Veterinary Medicine Department of Food Hygiene and Technology, ORCID: 0000-0002-8578-9623

1. The Importance of Species Identification in Meat

Determining the origin of the meat species in meat and meat products is extremely important in terms of health, economic, ethical, moral, religious and legal aspects. In order to reduce the production costs of meat and meat products, which is a subject we have encountered frequently in recent years, pork, donkey meat, etc. is added to beef. The negativities such as the addition of meat and the addition of cheap meat undermine the trust in foodstuffs. Due to the fact that pork and poultry meat is cheaper than beef, illegal production is made by mixing it into products made from red meat. It has been reported that food falsification in the meat industry has increased in recent years worldwide (Araç et al., 2022).

Consumption of fraudulent meat or meat products may cause the following problems for the consumer;

a. Different kinds of animal meats are offered for consumption without relying on religion and ethical considerations, and the consumer is deceived. For example; Muslim and Jewish consumers do not consume pork and pork products due to their religious beliefs.

b. Some people may be sensitive to meat and products of certain animal species and allergic reactions may occur.

c. Some animal meats (donkey, mule, dog, cat, crow, seagull, pigeon, etc.) that are not consumed by the society are offered for sale in the form of quality and meat-consumed animal meat, thus deceiving the consumer.

d. Since some animal meats (donkey, mule, dog, cat, crow, seagull, pigeon, etc.) that are not consumed by the society are slaughtered in an unauthorized and uncontrolled manner, various diseases spread.

2. Species Detection Methods in Meat and Meat Products

Species discrimination in meat and meat products is made by various methods. It is reported that methods based on sensory qualities, anatomical differences, histological features, characteristics of tissues and fats and glycogen amount, as well as immunological, electrophoretic and serological, genetic and spectrometric methods are used for species discrimination in meat and meat products (Özşensoy and Şahin, 2016).

2.1. According to Their Sensory Qualities

It is made on the basis of factors such as the specific color, smell, appearance, size and shape of the carcass in terms of sensory aspects. For example, when pork is evaluated sensorially, pork contains high intramuscular fat, has a urine-like odor and exhibits a soft consistency. The appear-

ance of meat and meat products, which is one of the sensory features, is perceived as quality for consumers and significantly affects purchasing. However, it has disadvantages such as being subjective of sensory qualities and not being made in minced meat, meat products, shredded body and meat. Some Characteristics of meat according to animal types are given in Table 1.

Table 1. *Characteristics of Meat According to Animal Species (Uğur et al., 1995)*

Cattle	Yellowish-white oily, finely fibrous, flesh light brick reddish.
Pig	It is harder, white, soft oily, and the color of the meat becomes lighter when cooked compared to the meat of other animals.
Sheep	There is a layer of fat under the skin, the flesh is light colored and thin.
Goat	It has a darker color, thicker structure, typical goat odor compared to sheep.
Horse	Very dark red or blue red color, turns rust color after contact with air, adipose tissue is dark yellow and there is no marbleization in muscle, adipose tissue is interfibrillar adipose tissue.
buffalo	Dark red and thick fibrous, fat is very white in color compared to cattle.

2.2. According to Their Anatomical Structures

Anatomically, a distinction can be made by considering the differences in bones and organs. The type of animal can be determined by making use of the anatomical features before the carcass is disintegrated. The disadvantage of this method is that it cannot be applied to small pieces of meat, minced meat or meat made into products. It also requires a good knowledge of anatomy. Today, this method has lost its importance as the mortar meat, whose meat is not consumed by the society, is converted into cubed, minced meat and/or meat products and sold. Sensory and anatomical differences between sheep and goat carcasses are given in Table 2.

Table 2: *Sensory and Anatomical Differences Between Sheep and Goat Carcasses (Arslan, 2020).*

Qualification	**Male**	**Female**
Smell	Mild ammonia odor	Distinctive goat scent
Colour	Red	Dark red
Structure of the trunk	Body cylindrical or slightly rounded	Flattened on the sides and longer on the back
Fatty and stickiness of the body surface	The surface of the body is fatty (especially the waist and tail area) and less sticky	The surface of its body is fatty-free and sticky
Length of the femur	Short	Long
fossa lacrimalis	There is	no
Structure of the scapula	It is broad, flat and short.	Narrow, steep and long
Tuberositase spina	There is	no
Number of vertebra caudalis	18-24 pieces	12 pieces
Foramen transversus	There is	no
Condition of the process spinalis	There is	no

2.3. According to The Amount of Glycogen

A distinction is made on the basis of the amount of glycogen in the meat. The amount of glycogen in meat varies according to the degree of formation of rigor mortis. Fresh horse meat contains higher levels of glycogen than other types of fresh meat, and pork liver contains higher levels of glycogen than the liver of other species. Again, the rate of glycogen in the fetus is high. However, the same type of animal meat has disadvantages such as different glycogen amount depending on the pre-slaughter processes, post-slaughter storage temperature and the hanging shape of the carcasses, and the determination of the glycogen amount in the products with various additives (Kang'ethe et al., 1982).

2.4. According to Fatty Analysis

Distinction of meat types is made by determining various properties such as melting and freezing points of meat fats, refraction indices, types and amounts of fatty acids, iodine, Reichert Meissl numbers. However, these tests have disadvantages such as, not being applicable to all products, the fact that the values in species are very close to each other, the oils in

different parts of the same animal have different properties, vegetable oils are mixed into the product (Kang'ethe et al., 1982). Although horse fatty contains 1-2% linoleic acid, the linoleic acid ratio in fatty of other species cannot exceed 0.1%. Depending on the amount of unsaturated fatty acids contained in the fatty, the iodine number increases. Because unsaturated fatty acids absorb iodine. The iodine number varies between 71-86 in horse fat, 38-46 in adult cattle, 35-46 in sheep and 50-70 in pigs.

2.5. According to Histological Structures

Only in crustaceans can species distinction be made according to their histological structures. In the meat distinction of other animal species, species distinction can be made by looking at the histological structure of the hair. Because hair structure differs histologically between animal species. The size and shape of the medulla cells, the thickness of the cortex, and most importantly, the shape, size and arrangement of the cells in the cuticle can be used in the distinction of meat species. However, the hair on the meat may not be from the animal from which the meat was obtained. Purposely, the hair of animals whose meat is consumed can be sprinkled on the meat of animals that are not consumed by the society and offered for sale. Or there may be no hair on the meat. Therefore, this method is also unreliable. In addition, it has been emphasized that these methods can be made in meat that has not been cut much and has not been subjected to any technological processing (Hofmann, 1986).

2.6. Species Separation Genetically

Species are differentiated on the basis of genetic differences between living things (differences in the sequence of purine and pyrimidine bases). Since DNA is a stable molecule that allows analysis of processed and heat-treated products, it offers the advantage of distinguishing between different animal species using DNA analysis. Some additional advantages of DNA-based assays include simplicity, speed, and specificity.

2.6.1. Hybridization methods

These methods are based on the detection of specific genes in tissue, organ, cell culture, secret and excrete genetic materials with labeled probes and their numerical amplification. Since some developments have been made in hybridization techniques in recent years, tests have been made easier, faster, more effective and reliable. When DNA hybridization techniques are examined, DNA in Southern Blot and Dot Blot hybridization techniques; It is seen that mRNA is used in the Northern Blot hybridization technique. The detection value for DNA hybridization is 0.1% to less than 0.01%, depending on the meat species (Derinöz et al., 2021).

2.6.2. Polymerase chain reaction (Polymerase Chain Reaction-PCR)

PCR is a technique based on the in vitro amplification of genetic materials (DNA or RNA) in a very short time (within a few hours). This method has been successfully used in the identification of species in living things (Kang, 2019). Its most important feature is the replication of target DNA. Methods based on DNA base sequence analysis are preferred in species identification studies because DNA is a more stable molecule compared to proteins, is less affected by high temperature, shows the same characteristics in all cells and tissues, and provides more information about the individual (Lockley and Bardsley, 2000). By using this method, meat type distinction can be made easily in a short time in fresh meats, pickled meats, meats with different degrees of heat treatment (boiled, roasted, fried, canned, etc.) and meat products.

More recently, biomolecular techniques such as PCR have received special attention. Some molecular techniques applied in the identification of meat species RAPD–PCR (Random amplified polymorphic DNA - Randomly amplified polymorphic DNA) (Lin et al., 2019), RFLP (restriction fragment length polymorphism) analysis (Guan et al., 2018), species-specific PCR (Vaithiyanatha et al., 2021), droplet digital PCR (ddPCR) (Košir et al., 2019), DNA barcoding method (Cottenet et al., 2020), real-time PCR (Chen et al., 2020) and multiplex PCR (Iqbal et al., 2020).

2.7. Immunological Methods

This method is based on antigen-antibody reaction. Since the antigens present in the tissues are different between species, a specific reaction occurs in antigen-antibody coupling. If one of the antigens and antibodies is evident, unknown antigens and antibodies can be detected due to the specificity of the reaction. Disadvantages of this method; Since meat belonging to genetically similar animal species have similar antigenic properties, cross-reactions occur and cannot be detected, and the species cannot be reliably distinguished by this method due to the denaturation of the antigenic structure in heat-treated meat and meat products, it is difficult to prepare the antisera used, the necessary antisera are not readily available in the market is absent (Zia et al., 2020).

The main methods developed for this purpose are; anaflaxie assay, precipitation method (Ring method, agar gel immunodiffusion (AGID) method), Immuno assay methods (Radio Immuno Assay (RIA) and Enzyme Linked Immuno Sorbent Assay (ELISA), immunosensors), meat species separation with rapid test kit

2.7.1. Anaflaxie trial

Antigenic substances belonging to animal species (meat extract, blood serum, blood) are injected into experimental animals to form antibodies. When these foreign proteins are given to the experimental animal twice, anaflaxie symptoms occur in the second injection. However, the disadvantage of this method is that this phenomenon can also occur with heterologous proteins.

2.7.2. Precipitation method

In this method, as a result of injection of foreign proteins (meat extract, blood serum, blood) to the experimental animals, antibodies are formed against this protein used as antigen in the experimental animal organism.

2.7.2.1. Ring method (Uhlenhut Method)

It is the formation of precipitation on the contact surface of two liquids by placing an antibody on the antigen in a tube to determine the presence of homologous antigen against the known antibody. An extract is prepared from the meat or meat product to be examined. From this prepared extract, rabbits are injected intraperitoneally with 5, 10 and 15 ml on the 1st, 2nd and 3rd days, respectively. On the 12th day following the last injection, blood is taken from the rabbits under aseptic conditions, the antiserum is removed, 0.5 ml of the meat extract to be examined is dipped into the bottom of the uhlenhut tube and slowly poured into it. The antiserum with a high density remains at the bottom. If the antigen and antiserum are homologous, precipitation occurs on the contact surface of the two liquids within 15 minutes. The disadvantage of this method is that the meat of species that are close to each other and meat mixtures below 10% cannot be determined exactly

2.7.2.2. Agar jel immünodifüzyon (AGID) yöntemi

Taking advantage of the diffusion ability of solidified agar, the antibody precipitates with the antigen. In this method, mutually separate wells are opened on the agar. Meat extract is added to one of these wells and antibodies are added to the other. Then the antigen and antibody move towards each other in the agar gel and precipitation occurs at the junction points. This method gives results in 72 hours. Disadvantages of this method; Cross-reactions between closely related species occur, are costly, and experiments take a long time.

2.7.3. Immuno assay methods

It is performed with Radio Immuno Assay (RIA) and Enzyme Linked Immuno Sorbent Assay (ELISA). The RIA method is based on the binding of radioisotope-labeled antibodies to the antigen-antibody complex in the solid phase and measurement with a gamma-counter device. The ELISA method, on the other hand, is based on the principle of binding specific antibodies to some determinants of the antigen, and enzyme-labeled antibodies to other determinant groups, and measuring the enzyme activity level by photocolorimeter through the substrate. ELISA is the most widely used immunoassay method for detecting meat adulteration (Ha, Thienes et al., 2018). In the ELISA method, two types of antibodies are used, namely, species-specific polyclonal and monoclonal antibodies. Commonly used ELISA methods for meat adulteration detection are direct ELISA (Seddaoui and Amine, 2020), sandwich ELISA (Thienes et al., 2018), and indirect competitive ELISA (Mandli et al., 2018).

ELISA is superior to other immunological and electrophoretic methods because its method is used on frozen products or products that have been heat treated at 80 °C for 60 minutes. However, the disadvantages of this method are; It is expensive, the use of monoclonal antisera is necessary, and it is expensive and time-consuming to sensitize and monospecific antisera.

2.7.3.1. Immunosensors

Since the ELISA method has its stated disadvantages, the researchers are dedicated to developing more sensitive, time-saving and low-cost protein-based methods to reveal meat cheats. Recently, it has been reported that immune sensors identify food adulteration detection (Ruiz-Valdepeñas Montiel et al., 2019). The principle of immunosensor methods is similar to that of ELISA methods, but it uses a biosensor, so the sensitivity of the method is better than that. In one study, using an electrochemical competitive immunosensor based on an anti-swine IgG polyclonal antibody, rates as low as 0.01% swine adulteration were detected within 20 minutes. According to this study ELISA, detection limit and detection time are reported to be greatly improved (Mandli et al., 2018). Although the immunosensor method is not widely used at the moment, it is promising for meat species determination, especially for on-site monitoring in the factory.

2.7.4. Separation of meat types with rapid test kit

It is a qualitative test method using test tubes containing anti-albumin antibodies and gives results in mixtures of 2% and above.

2.8. Protein-Based Methods

Protein is the main component of meat. The specific protein composition and the three-dimensional structure of specific proteins have certain protection and specificity among species suitable for meat adulteration detection. Also, some protein molecules are tissue-specific and can be used for the identification of less valuable additives such as connective tissue, blood plasma or milk preparations (Jiang et al., 2018). Protein-based methods include sodium lauryl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).) (Wang et al., 2022), isoelectric focusing (IEF) (Saud et al., 2019), ELISA (Thienes et al., 2019), Lateral flow device (LFD) (Magiati et al., 2019) and High Performance Liquid Chromatography (HPLC) (Häfner et al., 2021).

2.8.1. Detection of meat types by high performance liquid chromatography (HPLC)

Liquid chromatography-mass spectroscopy is generally used for determination of species-specific peptides in meat and meat products, metabolic, lipidomic and proteomic analyzes (Stachniuk et al., 2021). In meat and meat products, HPLC is used to detect soy proteins and identify some peptides. One of the most important advantages of the high-performance liquid chromatography method is that different detector types can be connected (Ishimaru et al., 2019; Häfner et al., 2021). In a study, hemoglobin iso-forms of different origin were separated by cation exchange chromatography by extracting hemoglobin from different meat species (cow, pork, lamb), and the peaks obtained with HPLC-DAD de-detector clearly distinguish pork from cow and lamb meat. data have been reported (Wissiak et al., 2003). While a maximum of seven species can be detected in the species determinations made by the chromatographic method, 13 different meat species (cattle, pork, goat, chicken, duck, ostrich, salmon, cod, crab, shrimp, etc.) clams, frogs and crocodiles) were identified (Chou et al., 2007). In another study, the determination of pork phospholipid molecular species was determined using high-performance liquid chromatography, tandem mass spectrometry and evaporative light scattering (Bosse-lini, 2008). Several high-performance liquid chromatography (HPLC) methods have been developed to analyze phospholipids at the molecular species level. It has been reported that phospholipid molecular species can be analyzed in a single step despite using smaller samples (Takahashi et al., 2018). An UPLC method for detecting the presence of pork in a raw beef burger was reported by Giaretta et al. (2013). According to this method, after pretreatment with sodium nitrite to convert oxymyoglobin and deoxymyoglobin in meat samples (beef, chicken, horse, ostrich, pig and buffalo) to more stable metmyoglobin, meat types were distinguished and identified.

2.8.2. Electrophoresis

In electrophoresis, species differentiation is made on the basis of the ability of proteins to move in an electrical field (electrical charges) in a support material at a certain pH or the differences in the molecular weights of proteins (Zia et al., 2020). Thus, specific protein bands (electrophoregamy) of each species meat types can be distinguished from each other. Separation can be accomplished by focusing on a single component, for example myoglobin, a muscle protein with relatively short chains.

For this purpose, agar gel, starch gel, sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and urea polyacrylamide gel are mostly used among the media that form the support phase on which proteins are applied and adhered to. In polyacrylamide gel electrophoresis (PAGE), molecules are separated according to both their size and electrical charge due to the pore structure in the gel. In SDS-PAGE, on the other hand, proteins are separated on the basis of their molecular size, since all of them will be negatively charged because they are processed with SDS.

Sodium-dodecyl-sulfate (SDS) was first reported to be used in electrophoresis by Shapiro in 1967. The use of the SDS gel electrophoresis technique was first used by Scopes and Penny (1971) to determine the animal species. Zerifi et al. (1991) used this method for various heat-treated animal meats and heat-treated pure horse meat; reported that they can easily separate it from beef, pork and mutton. With two-dimensional electrophoresis (2DE), it is also possible to identify meats of several related species simultaneously. Another advantage is that it can separate thousands of proteins simultaneously in a single gel.

Electrophoresis gives clearer results than immunological methods. In addition, cross reactions seen in immunological reactions do not occur. It can be used to distinguish mixed and shredded meat products, meats that have undergone certain degrees of heat treatment, as well as the amount of protein is determined quantitatively.

2.8.3. Isoelectrofocusing (IEF / Isoelectric Focusing)

The isoelectric focusing method is more sensitive than electrophoresis and it is stated that it is applied in fresh, chilled, heated, frozen meats, meat mixtures and products.

Isoelectric focusing was first used by Tingbergen and Olsman (1976) for animal species determination. In this method, support media are used, where the pH increases regularly from the anode to the cathode. In such an environment, if a protein is placed at a pH point lower than its isoelectric point, its electrical charge will be positive and will begin to move towards

the cathode. As the protein molecule moves towards the cathode, with the regular increase in pH, its electrical charge will begin to change, and at a certain isoelectric point it will be neutral and will not move. As soon as it moves from this point, it will be pushed back to its original place because it will gain an electric charge (Chappalwa et al., 2020). Tingbergen and Olsman (1976) used this method to determine the type of both raw and cooked meat products.

It is the migration of proteins to their isoelectric points where their net charge is zero with electric current in a support material (polyacrylamide gel) whose pH can vary between 2-11. Thus, the iso-electric points of different proteins are separated from each other by gathering in different places. It is emphasized that mixtures with a minimum of 2% can be detected. Bauer and Hoffman (1987) separated the meat proteins of various heat-treated animal species according to the isoelectric focusing method and dyed these gels according to the myoglobin staining method. They reported that with this method, it is possible to distinguish between meat types.

2.8.4. Lateral flow device (LFD)

The lateral flow analysis is based on an immunochromatographic (IC) procedure that uses the antigen-antibody reaction on a nitrocellulose membrane indicated by a color band of bound gold particles. The advantages of the method are that the analysis time is fast, a fast test time (<15 minutes), long-term stability in variable climatic conditions, cheap production, and being usable by untrained personnel without using special equipment. The disadvantage of this method is that some cross-reactivity may occur with non-target meats (Zia et al., 2020).

2.9. Spectroscopic Techniques

Spectroscopy is a fast and non-destructive technique for the verification of meat samples. The two most commonly used spectroscopic techniques for meat speciation are Infrared (IR) and Raman spectroscopy. Near infrared (NIR) spectroscopy, Laser-induced breakdown spectroscopy (LIBS), Hyperspectral imaging (HSI) and multispectral image (MSI) spectroscopy, Raman spectroscopy, Fourier transform infrared (FTIR) spectrometry methods are also used. Recently, NIRS was used to distinguish pure beef from pork-added ground beef with acceptable sensitivity and accuracy using PLS-DA models. Classification rates between 80% and 90% were obtained for validation sets (López-Maestresalas et al., 2019). Measurements in NIR spectroscopy are non-destructive and non-invasive. However, NIR raw spectra exhibit low specificity and do not show clear peaks specific to a particular compound of interest. In addition, extensive

statistical calculations are required to obtain useful qualitative or quantitative information.

2.10. Electronic Nose

Volatile compounds are another target method for meat species identification. Because each type of meat and meat products have special taste characteristics. Therefore, recently, the electronic nose method has been successfully developed for species detection in meat and meat products. Electronic noses (E-noses) are devices that are generally used to analyze food flavoring without separation and identification of volatile compounds. The advantages of this method are its simplicity, no special sample preparation, short analysis time and low cost per sample. The disadvantages of this method are that the metabolite content of the animal rearing environment, meat storage and processing conditions change, whether some metabolites are species-specific or not, and it does not give safe results. Therefore, this method is not widely applied at present (Zia et al., 2020).

3. Gender Detection in Meat and Meat Products

Meat quality is classified by considering factors such as the sex of the animal, age, fattening status and marbleization degree. Female animal meats are of lower quality compared to male animal meats, especially according to quality standards such as carcass weight, degree of muscle gain (muscle ratio), marbleization and fattening score. Meat of sterilized male animals is generally more valuable than the meat of cows of the same age. Depending on age, the length and stiffness of muscle fibers increase (increased connective tissue, especially collagen) and accordingly the quality of meat decreases. In many meat packaging plants, female animal meat and meat products are sold under the name of male animal meat or as a product made from male animal meat, and the consumer is deceived. Besides, gendering of beef has always been in the public interest in countries such as India, where the slaughter of cows (female cattle) is prohibited due to religious beliefs and therefore laws. It is necessary to have reliable methods for determining the gender of meat in order to avoid unfair competition and to reassure consumers about correct labeling (Gokulakrishnan et al., 2015).

Sex determination in the carcass can be made according to some anatomical and visual differences. However, there may be errors during the examination, especially when the visual differences are examined subjectively. Therefore, objective methods such as immunochemical and molecular techniques should be used for sex determination. Sensory and anatomical differences between male and female bovine carcasses are given in Table 3.

Table 3: Sensory and Anatomical Differences Between Male and Female Bovine Carcasses (Arslan, 2020)

Qualification	Male	Female
Body structure	In males, the carcass has a more rounded appearance, the muscles are thicker and coarser, especially in the neck and hind thighs, musculature is more pronounced.	In females, the carcass is straight and long, generally thinner, less muscular.
Neck muscles	Significantly developed and intense muscularity in men	It is underdeveloped, slender and thin in the female.
Lower branch of Os.ischii	Short and sunken inward.	Long and straight in the female
Color of meat	Red-dark	Red-pale
Oil color	White	Yellowish white, lemon yellow, especially in older females, the yellow color attracts attention.
Tuberculum pubicum	Large and overdeveloped in males	Teeth are small and flat
Cross-sectional surface of the gracilis muscle	Close to triangular shape in men	Bean or half moon shape
Chromosome difference	xy	xx

3.1. Determination of sex determination in meat and meat products by immunochemical and molecular methods.

To date, a number of different methodologies have been developed to determine the sex of meat, mainly based on the detection of hormone or DNA (Zeleny and Schimmel, 2002). Gas chromatography-mass spectrometry (GC-MS) (Hartwig et al., 1997), high-performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) (Draisci et al., 2000) and enzyme-linked immunosorbent assay (ELISA) (Simontacchi et al., 1999) have been found to be effective for measuring bovine sex hormones and thus determining sex. However, with the development of the polymerase chain reaction (PCR) method, PCR-based molecular diagnostic tests have been used in recent years because it is easy and cheap

for sex determination in meat and meat products. PCR-based studies using genomic DNA, amelogen (AMELX and AMELY) and zinc finger genes (ZFX and ZFY), Microsatellite and Y-chromosome-specific Sequences, testis-specific protein, y-coded (TSPY) gene, DEAD box polypeptide 3 is based on the principle of revealing X-linked/Y-linked (DDX3X/DDX3Y) gene entities. In addition, it has been stated that real-time PCR, SYBR Green and TagMan technology can be used in sex determination (Gokulakrishnan et al., 2015; Ahmad et al., 2021).

PCR-based methods have proven to be reliable tools for sexing meat and meat products. Precise results are usually available in as little as a few hours. Hormone analysis with GC-MS for sex discrimination is a valuable method, but this method cannot compete with PCR-based methods in terms of low cost and rapid availability of results.

4. Conclusion

Increasing demand for animal protein and increasing consumer awareness about the composition of meat make it essential to verify the source of meat correctly. In the past, researchers have used various methodologies for species and sex identification in meats. Today, with the development of technology, new, fast, easy, cheap and applicable methods have been developed. However, most of these methods seem to have disadvantages in terms of applicability. In addition, there is no single method that can meet all requirements for all sample types. It is hoped that in the near future new methods will be discovered fort he identification of a reliable and authentic meat product.

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CHAPTER 2

THE USE OF BLACK SOLDIER FLY (*Hermetia illucens*) LARVAE IN POULTRY NUTRITION

Onur KESER[1]

1 Araş. Gör. Dr., Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Animal Nutrition & Nutritional Diseases, Istanbul, TURKEY, ORCID ID: 0000-0001-8380-5549

Introduction

Protein is essential to have a sufficient level together with other nutrients in the ration to obtain the growth and yield performance expected from farm animals. In other words, while a nutrient-balanced diet is essential for growth, development and performance in animals, feed components in the diet should also have a high protein content, a balanced amino acid profile, high digestibility and palatability (Gałęcki et al., 2021). It is obvious that in the future, in parallel with the increasing world population, people's demand for animal products will increase and therefore there will be serious problems in the supply of feed and feedstuffs in the livestock sector trying to meet this demand. As a matter of fact, it is predicted by The Food and Agriculture Organization of United Nations that the world population will increase from 6.8 billion to approximately 9 billion until 2050, and the food requirement will increase by 70-100%, whereas agricultural production will only increase by 60% (FAO, 2011; FAO, 2014). It is also estimated that there will be 67%, 42%, 38% and 55% scarcity in corn, rice, wheat and soybean production in the future, respectively (Ray et al., 2013).

Feed cost constitutes 50-70% of the total cost in animal production (Spring, 2013), and the protein sources used constitute 25% of this cost (Albino et al., 1992). Among feedstuffs, protein supplements are the most expensive and limiting. Soybean meal (SBM) is considered to be a high-quality vegetable protein supplement used in animal rations, and 97% of soybeans produced worldwide are used as animal feed annually (Mottet et al. , 2017). Similarly, fishmeal (FM) is also used in animal nutrition as a high quality and digestible feed ingredient (Miles and Chapman, 2021). However, it has been reported that the production of fishmeal and oil has become increasingly limited due to overfishing to meet human food demand (Tacon and Metian, 2008), in the future, the use of protein obtained from human-edible soybean, other oilseeds and grains in animal feeding may be seen as a competitor to human food, however, feed supply may be affected by various factors such as the current status of arable land and water, climate change, energy cost, and government policies (Kim et al., 2019). Therefore, studies on alternative protein supplements in the field of animal nutrition have gained momentum in recent years and in this context, interest in insect larvae as a sustainable protein source has increased.

According to the European Food Safety Authority Scientific Committee, insect species that can be processed as animal feed include the black soldier fly (Hermetia illucens) in larvae, pre-pupae and pupae stages, house fly (Musca domestica) larvae for fish, chicken and pigs, mealworm (Tenebrio molitor) and lesser mealworm (Alphitobius diaperinus) as live larvae for pet animals, house cricket (Archeta domesticus), banded cricket (Gryllodes sigillatus) and field cricket (Gryllus assimilis) as live mature for

pet animals (EFSA, 2015). Protein sources must meet certain conditions in order to be used in animal nutrition. Among them; regularly available, economically valuable, non-competitive to human resources and environmental sustainability. In this context, it was reported that black soldier fly (BSF) larvae met these criteria and also contained fatty acids, polysaccharides and other possible components with nutritional value in addition to protein (Zulkifli et al., 2022).

The poultry industry, which has a great impact on meeting the daily protein requirement per capita worldwide and has a greater contribution to meeting this compared to pork and beef, is a very important sector in the production and procurement of protein-rich nutritious foods such as poultry meat and eggs (Dörper et al., 2021). Since 50-65% of the feed used in poultry production consists of energy and 25-40% protein sources, energy and protein sources constitute a large part of the cost in poultry feeds, therefore, the way to reduce the cost of feed depends on either supplying economic resources or finding alternative sources (İnal and Kahraman, 2015). In poultry production, more than 65% of the cost is feed, and more than 90% of the feed cost is meeting the energy and amino acid requirements (Kiarie et al., 2013).

SBM, FM and processed animal proteins are largely used as the main protein source in intensive poultry nutrition, but there is an increasing decrease in soybean cultivated lands and fish stocks around the world (Midilli and Özcan, 2020). In addition, it is an inevitable fact that problems such as land and water scarcity, food-feed-fuel competition and climate change will be the main obstacles for the sustainability of these protein sources in the future (Van Huis et al., 2015). In this context, finding cheap and sustainable new protein sources in the poultry industry is essential in order to provide people with cheaper access to animal protein (Özek, 2016), and BSF larvae attracted attention as a promising source in this regard with their rich protein and fat content on the basis of dry matter and appropriate amino acid profile (Gold et al., 2018). In this section, it was aimed to present general information on the use of black soldier fly larvae in animal nutrition and to mention the results of studies on this subject in recent years.

General Information on Black Soldier Fly and Its Larvae

Black soldier flies are a common fly of the family *Stratiomyidae* and are very common mostly in the Western Hemisphere and Australia. This fly species, which generally spreads from the tropics to the warm climates throughout the world, leads an active life from April to October in the Southwestern Regions of the United States (Park, 2016; Tomberlin and Sheppard, 2002). There are 76 species of the genus *Hermetia*, of which 39 are located in the Neotropical region and 20 are distributed in vari-

ous regions. In these species, only the larvae of four species are known: *Hermetia albitarsis*, which is distributed in Brazil, *Hermetia aurata* and *Hermetia concinna*, which are distributed in Mexico, and *Hermetia illucens*, which has a cosmopolitan distribution (Barros-Cordeiro et al., 2014). Among them, *Hermetia illucens* is considered as a promising species in the insect-farming industry (Tomberlin and van Huis, 2020). It has been reported that *Hermetia illucens*, which is thought to be of American origin, spread to the world through trade route and can be seen outside of the tropic and subtropic regions (West Palacartctic and Near East) because it can tolerate climatic conditions (Üstüner et al., 2003). However, since they are vulnerable to cold, this situation prevents their invasion to regions such as Northern Europe (Spranghers et al., 2017a).

The length of an adult black soldier fly ranges from 15 to 20 mm, and the wing size of females is larger than that of males (Sheppard et al., 2002; Gobbi et al., 2013). Adult forms do not need to eat anything except for water cosumption, and their survival depends entirely on the fat stored in their body during the larval period. Although they do not have the risk of polluting the environment, spreading diseases and damaging crops, they prefer to stay away from people and to shady areas for mating (Liu et al., 2019). Adult females start to lay eggs about 2-3 days after mating and egg production can reach up to 900 eggs per female (May, 1961). The larvae that emerge from the eggs within 4 days begin to live as scavengers on decaying plant materials such as fruit, vegetables, corn, coffee pods and animal materials such as human and animal feces and cadavers (Rozkosny, 1983). The larval stage of black soldier fly consists of 6 instars (Hall and Gerhardt, 2002). Biomass accumulation in larvae varies between 14-22 days depending on the environmental conditions and feeding conditions, and in the pre-pupa period, which is the last stage of the larval period, the larva quits feeding and forms pupae on a dry surface. It can take a total of 40-43 days for black soldier flies to transition from birth to adulthood (Wong et al.,2019). It has been reported that the suitable ambient temperature for BSF larvae is 24-30°C, the optimum ambient temperature is 26-27°C, temperature above 30°C causes the larvae to flee to cooler parts, and temperatures below 24°C causes a decrease in their metabolic activities, they can tolerate the moisture content of the substrate used for rearing them in the range of 60-90%, optimum substrate moisture content is recommended to be between 60-70% (Shit, 2021; Dortmans et al., 2017; Holmes et al., 2012). However, in a study on threshold temperature and thermal requirement in black soldier flies, it was observed that hatching rate, larval survival rate, prepupal survival rate, and black soldier fly fecundity were highest at 30, 35, 35 and 30°C, respectively (Chia et al. al., 2018). The life cycle of the black soldier fly was shown in Figure 1.

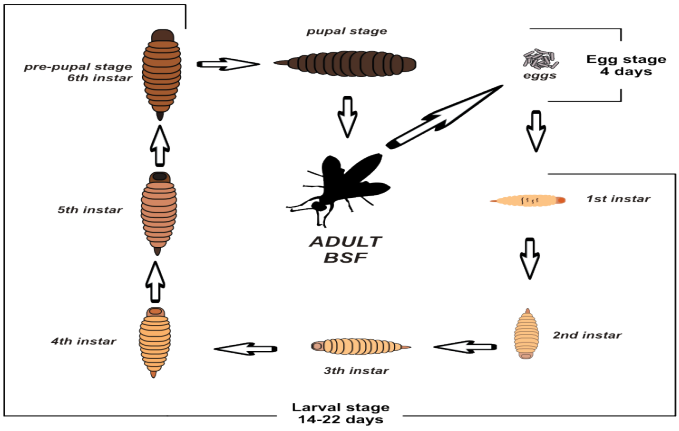

Figure 1. *The life cycle of the black soldier fly (Hermetia illucens)*

The advantages of the production and use of black soldier fly larvae as a feed can be listed as follows;

I. BSF larvae can play an important role in the management of organic waste: It has been reported that BSF larvae, which attract attention in the recycling of biological wastes, are a potential recycler in the evaluation of various organic wastes such as slaughterhouse, food, fruit-vegetable and human waste, and that the larvae can convert 4 kg of organic waste into 800 g larval biomass under appropriate conditions (Lu et al., 2021; Ibadurrohman et al., 2020). Only in fruit and vegetable wastes, 125 kg of fresh and 40 kg of dry BSF larvae can be obtained from 1000 kg of waste in 14-21 days (Ravi et al., 2020). In addition, the gluttonous nature and strong mouth structure of BSF larvae increase the rate of decomposition of organic wastes, and their long life cycle makes them more effective in the bioconversion process than other fly species such as Musca domestica and Lucilla cericata (Oliviera et al., 2015; Cickova et al., 2014). In a review written by Salam et al. (2021), it was stated that the reduction rate of BSF larvae for municipal waste, human excreta, pig manure and chicken manure was 68%, 73%, 39% and 50%, respectively.

II. Emissions of carbon-based greenhouse gases are low: The ability of BSF larvae to recycle food waste ensures that 70% less carbon-based

greenhouse gases are released into the atmosphere compared to the composting of these wastes by microbial decomposition process, and they do this by capturing the high amount of carbon in the wastes and storing them in the form of protein, oil and chitin, which have high economic value and potential (Perednia et al., 2017).

III. They can reduce the pathogen load in waste: BSF larvae can reduce the microbial load in the environment by eating the bacteria in the substrate and killing bacterial colonies through high pH conditions and enzymatic reactions in their digestive systems, and competitive exclusion of the bacterial flora in their gut with harmful bacteria (Gold et al., 2018). It has been reported that BSF larvae can also reduce up to 93% of pathogenic bacterial species such as *Clostridium*, *Eschericia coli*, *Enterococcus*, *Salmonella*, which threaten the environment and human health, in animal feces (Awasthi et al., 2020). Gorrens et al. (2021) observed that when *S. aureus* was inoculated into chicken feed which used as substrate in BSF larvae, this pathogen was not detected in the larvae and the *S. aureus* load in the substrate decreased significantly after 6 days.

IV. They generate useful waste as soil fertilizer in agricultural production: Insect excrement (frass) produced during the consumption of animal manure and other wastes by BSF larvae can be considered as high quality fertilizer and this allows the recycling of natural nutrients and the reduction of the loss of important nutrients in the soil (Newton et al., 2005, Bortolini et al., 2020). Under appropriate conditions, 1000 kg of fruit and vegetable waste can be converted into 250 kg of frass in 14-21 days by BSF larvae (Ravi et al., 2020).

V. They have high nutritional value: Due to their high protein, balanced essential amino acid profile and rich saturated fatty acids content, BSF larvae are an acceptable food for many animal species (Sevilmis et al., 2019). Although other insect species such as mealworm larvae (*Tenebrio milotor* L.) and crickets (*Ortophera: Gryllidae*) have also been shown as alternative feed sources for poultry and pigs, the better feed conversion rate, the high survival rate, and the stable nitrogen and phosphorus composition against dietary changes (Schiavone et al., 2017; Gasco et al., 2019; Oonincx et al., 2015) makes BSF larvae more advantageous than other insect larvae.

Nutrient Composition of Black Soldier Fly Larvae

Comprehensive biochemical analysis of feedstuffs is an important issue in terms of nutrient values, and the nutrient composition of insects varies according to the methods of farming and processing as feed material (Fasakin et al., 2003). Considering that feed materials with a crude protein

ratio of more than 19% are classified as a protein source, it was reported that BSF larvae were an insect type that could be used as an alternative protein source that could meet this requirement (Mahmud et al., 2020). It was reported that the feed conversion ratio of BSF larvae reared on various organic wastes ranged from 1.4 to 2.6 and could convert these substrates into high quality protein, and the larvae had an amino acid composition rich in methionine and lysine (Oonincx et al., 2015). BSF larvae have a high-value feedstuff potential because they are easy to breed, fast growing, efficiently converting organic waste, rich in protein and even have a better amino acid profile than soybean meal, as well as containing high amounts of lipids, calcium, phosphorus, sodium and magnesium (Berragan-Fonseca et al., 2017; Chia et al., 2019). It has been reported that BSF larvae contain high levels of lysine, methionine and threonine amino acids, which are known as limiting amino acids for poultry fed soybean and corn based diet, have a higher content in terms of leucine, lysine and arginine amino acids compared to corn gluten meal, and they are rich in alanine, methionine, histidine and tryptophan compared to SBM (Shah and Çetingül, 2022; Barragan-Fonseca et al., 2017). BSF larvae are rich in saturated fatty acids, and lauric acid (C12:0) is the most abundant, followed by myristic (C14:0), palmitic (C16:0) and stearic acid (C18:0). Oleic acid (c9C18:1) is the highest monounsaturated fatty acid and it is followed by palmitoleic (C16:1), linoleic (C18:2n6) and liolenic acid (C18:3n3) (Lu et al., 2022). BSF larvae are reported to contain approximately 31% protein, 26% fat (saturated fat, MUFA and PUFA content of 21%, 3 and 2), 23% carbohydrates (17% NDF and 6% other), 13% ash and 6% chitin (Ravi et al., 2020), and these values may vary under the influence of various factors. There is a high correlation between the crude fat ratio in the larvae and the non-fiber carbohydrates (NFC) in the substrate, as lipids containing various fatty acid forms are synthesized through glycolysis, oxidative decarboxylation and then fatty acid synthesis cycle from NFC in the substrates on which the larvae are fed (Ewald et al., 2020; Spranghers et al., 2017b). The presence of non-digestible chitin, a polysaccharide composed of N-acetylglucosamine units containing a nitrogen atom, may overestimate the crude protein content of the larvae (Jonas-Levi and Martinez, 2017). Therefore, it is recommended to use 4.67 instead of 6.25 as a nitrogen to protein conversion factor to prevent the contribution of chitin to total nitrogen when calculating crude protein in BSF prepupae by Kjeldahl method (Janssen et al., 2017). The proximate nutrient contents, amino acid composition and fatty acid composition of BSF larvae are presented in Table 1, 2 and 3, respectively.

Table 1. *Proximate composition of BSF larvae (% of dry matter)*

Reference	(1)[f/m/c]	(2)[L/Pp/P]	(3)[pd/hd]	(4)	(5)	(6)[ff/d]
Dry matter, %	96.5/96.4/97.6	32.0/35.0/40.0	94.2/98.5	94.7	36.3	95.9/93.5
Crude protein	54.9/55.7/55.9	39.0/37.0/36.0	55.3/65.5	39.8	44.3	45.8/56.1
Crude fat	21.5/20.2/14.7	28.0/34.0/28.0	18.0/4.6	24.3	31.9	25.8/4.9
Crude fiber	-	-	-	9.6	5.1	-
Ash	13.6/13.5/11.3	10.0/10.0/14.0	9.9/9.3	14.7	-	6.9/11.4
Chitin	1.8/2.7/15.4	4.8/7.2/6.3	5.0/6.9	-	-	-
Carbohydrate	-	-	-	-	3.4	17.4/21.2
Gross energy, MJ/kg	22.4/22.1/21.6	-	24.4/21.2	21.7	25.5	-

[f/m/c] Particle size: fine (0-200 µm), medium (200-400 µm) and coarse (>400 µm), respectively; [L/Pp/P] Values for larvae, prepupae and pupae, respectively; [pd/hd] Values for partially defatted and highly defatted larvae, respectively; [ff/d] Values for fullfat and defatted larvae, respectively; (1): Eggink et al. 2022b; (2): Weththasinghe et al. 2022; (3): Schiavone et al. 2017; (4): Sudha et al. 2022; (5): Bbosa et al. 2019; (6): Zozo et al. 2022.

Table 2. *Amino acid composition of BSF larvae (% of crude protein)*

Reference	(1)	(2)	(3)	(4)	(5)[L/Pp/P]	(6)
Arginine	6.1	5.6	2.1	5.5	4.8/4.8/5.5	4.8
Histidine	2.7	-	1.5	3.3	2.7/3.0/3.4	3.3
Isoleucine	4.7	19.0	4.5	4.7	4.2/4.2/4.7	4.2
Leucine	7.1	24.0	5.0	7.8	6.6/6.6/1.4	6.7
Lysine	6.0	3.9	4.0	6.8	5.9/5.6/6.4	5.9
Methionine	1.7	26.3	1.2	2.1	1.7/1.7/1.7	1.6
Phenylalanine	4.6	7.7	2.4	4.8	3.9/3.8/4.1	3.6
Threonine	4.1	-	2.2	4.4	3.8/3.8/4.3	3.9
Tryptophan	-	-	-	-	1.8/1.4/1.6	-
Valine	6.4	3.2	6.2	6.8	5.8/5.8/6.8	5.7
Alanine	6.9	-	5.9	8.2	6.4/6.0/6.8	7.8
Aspartic acid	8.5	-	5.2	7.3	8.6/8.3/11.0	8.2
Glycine	5.2	-	4.0	6.2	5.1/5.1/6.5	5.6
Glutamic acid	8.7	4.6	8.1	13.1	11.0/9.9/11.0	11.8
Cysteine	-	-	-	0.8	0.8/1.1/0.8	0.9
Tyrosine	7.1	9.7	2.7	6.7	5.7/5.9/6.8	5.1
Proline	5.5	7.0	6.2	6.7	5.6/5.2/6.2	6.2
Serine	3.9	3.5	2.7	4.9	4.2/4.1/4.7	4.3

[L/Pp/P] Values for larvae, prepupae and pupae, respectively; (1): St-Hilaire et al. 2007; (2): Bbosa et al. 2019; (3): Queiroz et al. 2021; (4): Mikołajczak et al. 2023; (5): Weththasinghe et al. 2022; (6): Tschirner & Simon, 2015;

Table 3. *Fatty acid composition of BSF larvae (% of total fatty acids)*

Reference	(1)	(2)	(3)	(4) $^{L/Pp/P}$	(5) $^{ViL/Pp}$
Capric acid (C10:0)	1.6	0.9	0.8	-	0.5/1.2
Lauric acid (C12:0)	58.9	46.0	39.5	37.0/43.0/65.0	28.1/61.9
Tridecanoic acid (C13:0)	0.1	-	-	-	-
Myristic acid (C14:0)	11.1	8.7	8.1	7.5/6.9/9.7	3.9/9.1
Myristoleic acid (cis-9 C14:1)	0.5	-	0.2	-	0.7/0.5
Pentadecanoic acid (C15:0)	0.3	0.2	0.1	-	0.8/0.0
Palmitic acid (C16:0)	12.7	12.2	14.5	16.0/13.0/8.6	5.8/7.9
Palmitoleic acid (cis-9 C16:1)	2.2	1.9	2.5	3.5/5.9/2.8	1.7/2.4
Margaric acid (C17:0)	0.1	0.2	0.1	-	33.6/3.2
Heptadecenoic acid (C17:1)	0.1	0.2	0.1	-	0.0/0.0
Stearic acid (C18:0)	1.2	2.5	2.3	2.9/1.8/1.2	0.7/1.2
Oleic acid (cis-9 C18:1)	7.4	11.2	10.1	-	4.3/5.3
Linoleic acid (cis-9,12 C18:2)	3.5	14.1	19.2	14.0/6.7/5.2	1.3/2.4
Eicosenoic acid (cis-11 C20:1)	0.3	0.1	-	-	8.0/0.0
Saturated fatty acids	86.0	70.7	65.5	-	73.5/84.5
Unsaturated fatty acids	13.8	-	-	-	-
Monounsaturated fatty acids	10.5	13.4	13.7	-	15.0/8.4
Polyunsaturated fatty acids	-	15.9	21.1	-	11.2/7.1

$^{L/Pp/P}$ Values for larvae, prepupae and pupae, respectively; $^{ViL/Pp}$ Values for V instar larvae and prepupae, respectively; (1): Nekrasov et al. 2022; (2): Daszkiewicz et al. 2022; (3): Zotte et al. 2018; (4): Weththasinghe et al. 2022; (5): Giannetto et al. 2020

Variables such as the harvest-age of larvae, the processing method, and the consumed substrates during rearing can be counted among the main factors affecting the nutrient composition of BSF larvae. Liu et al. (2017) reported that as the age of the larvae increased between 4-14 days, the protein content decreased to 38%, which is the minimum level, on the dry matter basis, while the fat content increased in the same period and reached the maximum level of 28.4%. Similarly, Rachmawati et al. (2010) found that the protein content of 61% on a dry matter basis in 5-day-old larvae decreased to 44% and 42% at 15 and 20 days of age, respectively, whereas 13% fat content on a dry matter basis at 5 days of age increased to 19% and 23% at 15 and 20 days of age, respectively. In a recent study carried out to investigate the changes in nutrient content in BSF larvae at different rearing periods, dry matter contents of larvae at 5, 10, 15, 20 and 25 days of age were 21.98%, 24.60, 25.56, 25.75, 30.47, crude fat contents were 22.89%, 24.82, 26.79, 33.40, 34.09 , crude fiber contents were 9.07%, 8.96, 9.29, 10.50, 10.40, crude protein contents were 49.9%, 44.43, 43.84, 41.58, 42.98, respectively, and it was reported that dry matter, crude fat and crude fiber contents tended to increase and crude protein content tended to decrease depending on the prolongation of the rearing period of

H. illucens larvae (Mahmud et al., 2020). Removing the fat from the larvae (defatting) also can change the protein level. Indeed, it was reported that defatting process in larvae containing 42.1% crude protein increased the crude protein content to 56.9% (Makkar et al., 2014). In a study by Schiavone et al. (2017), the dry matter content was 94.2% and 98.5%, the crude protein content (dry matter basis) was 55.3% and 65.5%, the chitin content was 5.0 and 6.9%, the gross energy was 24.4 and 21.2 MJ/kg for partially and highly defatted BSF larvae, respectively, and amino acid concentrations also increased depending on deffating level. The method of killing the larvae may affect the amino acid content. It has been reported that while freeze-killing activates the enzymatic pathways that lead to the loss of lysine and cysteine amino acids, the scalding does not have a negative effect on amino acid profile (Leni et al., 2019).

There are also studies investigating the effects of the substrate on which BSF larvae are grown on the larval nutrient composition. Studies on the use of biowaste and by-products such as animal excreta, brewery by-products, fish offal, fruit and vegetable wastes in the feeding of BSF larvae have shown that the larval nutrient composition has been modified by these substrates used in the rearing of larvae (Eggink et al., 2022a). In a study by Julita et al. (2018), BSF larvae were reared on horse manure, 50% horse manure + 50% vegetable waste, sheep manure and 50% sheep manure +50% vegetable waste, and it was determined that crude protein, crude fat and energy contents varied between 35.38%-46.59%, 10.97-14.09% and 4270-4603 Kcal/kg, respectively. It was concluded that the best result was obtained from the substrate made by half and half vegetable waste + horse manure, and it is remarkable that the most obvious change is in the crude protein content and the addition of vegetable waste to horse manure increased the prepupal crude protein content from 35.58% to 46.59% in larvae, while the addition of vegetable waste to sheep manure increased the crude protein content from 40.00% to 44.13%. In BSF larvae reared on 6 types of agricultural by-products (apple, banana, apple+banana [in 1:1 ratio], spent grain, apple+spent grain [in 1:1 ratio] and banana+spent grain [in 1:1 ratio]) as substrate, crude protein contents at the end of the trial were determined as 31.12%, 36.50, 35.60, 47.83, 48.01 and 45.61, and crude lipid contents were determined as 36.1%, 27.9, 33.4, 22.5, 20.1 and 23.1, respectively, and the crude protein contents in BSF larvae reared by using spent grain were significantly higher than those reared on the fruit diet alone (Scala et al., 2020). In the same study, no significant difference was found between the groups fed the spent grain diet in terms of crude lipid contens, but it was significantly lower than those fed only the apple diet. In a recent study carried out by Eggink et al. (2022a) reared BSF larvae on 6 different substrates (mixed feed [66.5% water, 16% pea grits, 8%

wheat, 7% chicken starter feed, 2.1% sugar beet pellet, 0.4% vitamin-mineral mixture], soybean meal-wheat based commercial chicken feed, rapeseed cake, brewer's spent grain, mitigation mussels [including tissues and shells]) and it was determined that the larvae fed with chicken feed had the highest crude fat content (32.9%), the larvae fed with mitigation mussels had the lowest crude fat content (21.6%), the larvae fed with chicken feed, rapeseed cake, and mixed feed had the highest energy content (23.2-24.1 kJ/g DM) and the larvae fed with mitigation mussels had the lowest energy content (17.7 kJ/g DM), however, in terms of amino acid profile, there was no significant correlation between the amino acids in larvae and the substrate. In terms of fatty acid profile, while the saturated fatty acid was more (47.5-60.8%) in the larvae fed chicken feed, mixed feed and brewer's spent grain, mono-unstatuated fatty acids were determined more (41.3-53.7%) in those fed with other substrate. In addition, it was determined that the content of omega-3 fatty acids in larvae fed with marine-based substrate was higher than the other groups, and they reported that the larvae accumulated omega-3 fatty acids from the substrate.

The correlation between the nutrient content of the substrates and the nutrient content of the larvae may vary. In a study by Shumo et al. (2019) on BSF larvae reared on chicken manure, kitchen waste and spent grain as substrate, it was reported there was a high correlation between the crude protein, crude fat and neutral detergent fiber contents of the larvae and the contents of the substrate. Similarly, in the study by Nguyen et al. (2015), the larvae were fed with standard poultry feed, pig liver, pig manure, kitchen waste, fruit+vegetable waste mixture, fish rendering waste, and it was reported that the larvae fed with fish waste and liver, which have the highest energy, calorie and fat content, also had a high calorie, protein, fat and carbohydrate contents. In contrast, Spranghers et al. (2017b) reported that although there were great differences in protein content between substrates, there were no significant differences in crude protein content between larvae fed these different substrates. Similarly, in a study by Adebayo et al. (2021), the larvae fed food remains with the highest crude protein ratio had the lowest crude protein content, while the larvae fed chicken feed with the lowest crude protein ratio had the highest crude protein content. According to a meta-analysis study carried out by Fitriana et al. (2021), the result of the statistical analysis of the data obtained from 43 studies derived from 13 articles demonstrated that crude protein and crude fat contents of BSF larvae were similar and not affected by substrates (animal feed, food waste, faeces and others).

The Effects of BSF Larvae on Growth and Yield Performance in Poultry

In poultry industry, parameters such as growth rate, feed efficiency and carcass characteristics are the important criteria in the evaluation of the growth performance of animals. As in other alternative feeds and feedstuffs, the efficacy of BSF larvae were also evaluated in terms of these parameters in several studies.

In a study, prepupae obtained from BSF larvae were dried, ground and added to a commercial broiler ration at a rate of 15%, and no negative results were found and when compared with the control group, similar results were obtained in terms of growth performance and carcass weight (Kinasih et al., 2018). In the same study, the hemoglobin concentration of the BSF prepupae group was significantly higher than the control group, and it was reported that this could help prevent anemia syndrome in poultry as a result of the improvement of iron utilization by BSF prepupae. Also, the addition of ground BSF prepupae to the ration did not change the ratio of water and crude protein in broiler meats compared to the control group, but significantly reduced the ratio of crude fat. In a study conducted by Onsongo (2017) to investigate the effects of the additon of BSF larvae meal at a rate of 0%, 5%, 10% and 15% on broiler performance and meat quality, BSF larvae meal was used as a substitute of SBM at a rate of 13.3%, 26.3%, 45.2% and as a substitute of FM at a rate of 14.0%, 30.0% and 35.0% for starter (day 7 to 28) period, and was used as a substitute of SBM at a rate of 19.0%, 46.0%, 64.0% and as a substitute of FM at a rate of 0%, 25.0%, 43.8% for finisher (day 20 to 49) period. As a result of the experiment, the addition of BSF larvae meal up to 15% to the diets had a similar effect with the control group in terms of body weight gain (BWG), average daily feed intake (ADFI), feed conversion ratio (FCR) and sensory characteristics of cooked breast meat in broilers. For starter and finisher periods, it was also determined that the replacement of BSF larvae meal with SBM at a rate of 45.2% and 64.0%, respectively, and the replacement with FM at a rate of 35.0% and 43.8%, respectively, created 11.4% and 10.3% reductions in the cost of diet for these two periods, respectively. In an other study carried out by Onsongo et al. (2018) in broilers, the effects of the replacement of BSF prepupae meal with SBM+FM mixture at a rate of 13.8%, 27.4% and 42% of the crude protein content in starter diet, and at a rate of 11.0%, 37.2% and 55.5% of the crude protein content in finisher diet, were investigated, and it was reported that there were no significant difference between the groups in terms of BWG, ADFI, FCR and breast meat aroma and flavor. Also, it was noted that the group fed the highest ratio of BSF prepupae meal had 16% more Cost Benefit Ratio and 25% better Return on Investment.

In broilers, control group was fed corn-SBM basal diet and experimental groups were fed diets supplemented with BSF larvae meal at a rate of 3% and 6% of control diet (Choi et al., 2013). As a result of the study, there were no significant differences among groups for live body weight (LBW), BWG, feed intake (FI) and percent of viability, and FCR was 1.57, 1.65 and 1.62 for control and experimental groups, respectively. In the same study, no difference was found between the groups in terms of carcass rate, abdominal fat and relative weights of thigh, wing, neck and back, however, the relative weight of breast meat increased significantly in the experimental groups compared to the control. Uushona conducted a study in which BSF prepupae meal supplemented at a rate of 0%, 5%, 10% and 15% to broiler diet, and they reported that there was no significant difference between the groups in terms of ADFI, BWG, FCR and European protein efficacy factor, organ weight, gizzard erosion score, tibia ash percentage, tibia breaking strength, tibia mineral content, small intestine pH, and duodenal and jejenal histomorphology. In the same study, it was also noted that the use of BSF prepupa meal in the broiler diet did not cause any physical, sensory or chemical quality defect in broiler meats.

In a study conducted by van Schoor (2017) to investigate the effects of the use of BSF prepupae meal, reared on human fecal waste and applied different washing processes (30 min at 62°C, 60 min at 62°C, 5 min at 72°C, 15 min at 72°C, 2 min at 100°C, 5 min at 100°C , rinsing in 5% propionic acid and rinsing in 5% formic acid), at a rate of 10% in broiler diet, it was reported that FCR, protein efficiency ratio, European protein efficiency factor and final live weight were better in BSF prepupae group than the control group, there was no significant difference between groups in terms of gut pH value and duodenum-jejenum histomorphology, more red breast meat was obtained in the experimental groups compared to the control group, however, the carcass yield in the experimental groups was significantly lower than the control group. In an experiment by Moula et al. (2018), 30-day-old Ardennaise chickens, a local breed, were fed the diet in which 8% of the diet was replaced by de-frozen BSF larvae obtained from larvae reared on horse manure, which corresponded to 2% of the diet on a dry matter basis. It was determined that there was no significant difference between control and experimental groups in terms of ADG, FCR, carcass weight, carcass yield, organ weights and tibia ash content. It was also reported that there was no significant difference in the control and experimental groups in terms of protein content, proportions of saturated (SFA) and mono-unsaturated fatty acids (MUFA) and omega6/omega3 ratio in pectoral muscle samples, but the overall proportion of polyunsaturated fatty acids (PUFA) in the groups fed BSF larvae was significantly higher than the control group. Dabbou et al. (2018) investigated the effects

of the supplementation of the partially defatted BSF larvae to the diet at increasing rates (0, 5, 10, and 15%) in broilers. It was stated that the addition of BSF larvae up to 10% in the starter period cused an increase in LBW, ADG, FI without affecting the FCR, and the increase in LBW and FI could be a result of the increase in the flavor of the diet by the addition of larvae, whereas the addition of BSF larvae at a rate of 15% in growing and finisher period adversely affected FCR and this might be due to the chitin content of the larvae.

In Barbary quails (*Alectoris barbara*), control group was fed SBM-based diet, esperimental groups were fed diet supplemented with BSF larvae meal to replace 25 and 50% of SBM, and at the end of the experiment (64 d), LBW and carcass weights of the experimental groups were significantly higher than the control group, in addition, although intestinal length and weight were lower than the control group, the nutrient digestibility was not affected (Loponte et al., 2017). The results of some recent studies in broilers are presented in Table 4.

In poultry production, egg yield and quality are also important parameters besides growth performance, carcass characteristics and meat quality. In addition to the efficient production of eggs, which have a very important place in meeting the protein requirements of the human population around the world, sufficient features in terms of quality criteria is also a very effective issue in the prevention of economic losses that may occur during and after the production phase.

Table 4. *Results of the recent studies on broiler performance*

Animal	Supplementation	Result	Ref.
Broiler (male Cobb) one-day-old n=360	Defatted BSFLM C, T1, T2, T3 %0, %4, %8, %12 Exp.period: 6 weeks	-C and T1 had higher FI during finisher stage, -T1 and T3 had lower FCR during finisher stage, -T1 had heaviest weight for 6-week feeding trial. It was concluded that %4 defatted BSFLM could be used to replace FM and SBM.	(1)
Broiler (Ross x Ross) one-day-old n=240	T1, T2, T3, T4, T5 T1: Casein diet (CP: %10) T2: SBM diet (CP: %12.8) T3: FM diet (CP: %9.5) T4: BSFLM diet (CP: %11.7) T5: BSFLM+EAA diet (CP: %14.9) Exp.period: 10 days	-T5 had greater BWG than other treatments, -T5 and T3 had greater FI than other treatments, -T5 had higher CPI than other treatments, -No differences amog treantments for PER, -No differences among T4, T3, T2 and T1 for BWG, -No differences among T4, T2 and T1 for FI, -No differences among T4, T3 and T1 for FCR, -No differences between T4 and T2 for FCR, It was concluded that the protein quality of BSFLM without or with additional EAA was comparable with FM and SBM.	(2)
Broiler (Ross 308) 4-day-old n=180	Live BSFL C, T1, T2 C: %0 larvae T1: %5 live BSFL T2: %5 live *T. molitor* larvae Exp.period: 39 days	-No differences between groups for BW, ADG and ADFI -No differences between C and T1 for FCR, -No differences between groups for relative weight of hot carcass, cold carcass, breast yield, thigh yield, liver, bursa of fabricius, heart, intestine, glandular stomach, gizzard, abdominal fat, caecal length, -T1 had higher spleen relative weight than C. It was concluded that the administration of live larvae had no negative effect on performance and health status.	(3)

Broiler (Ross 308) one-day-old n=380	Partial or total replacement of SBM with full-fat BSFLM C, T1, T2, T3 C: %0 replacement T1: %50 replacement T2: %75 replacement T3: %100 replacement Exp. period: 42 days	-LBW of T1, T2 and T3 were lower than C on day 14, 35 and 42. LBW of birds decreased with increasing dietary inclusion levels of BSFLM. A linear downward trend in final body weight was observed. -ADG of T1, T2 and T3 were lower than C between 1-14 d and between 14-35 d. Between d 35 and 42, no difference between C and T1 for ADG, however, T3 had the highest ADG. - ADFI of T1, T2 and T3 were lower than C at 1-14 d, 14-35 d and 35-42 d. -No difference among C, T1 and T2 for FCR during the entire experiment and more favorable than T3. -Experimental groups had lesser meat and more abdominal fat than C. The meat from T2 and T3 characterized lower juiciness and taste intensity than C and T1. The rate of autoxidation (mg MDA/kg) in breast muscle was lower in T1 than in C and other treatments. It was concluded that the inclusion rate of BSFLM as a substitute for SBM should not be exceeded 50%.	(4)
Broiler (Ross 308) one-day-old n=384	Full-fat BSFLM as a substitute of SBM C, T1, T2, T3 C: %0 substitution T1: %50 substitution T2: %75 substitution T3: %100 substitution Exp. period: 42 days	-In *Pectoralis major* muscle, total concentration of pigments were higher, ash contents were lower in BSFLM groups. -T1 had a lower fat and collagen content in *Pectoralis major* muscle than other groups. -Total SFA concentartions increased and total PUFA concentrations decreased in muscle with increasing inclusion levels of BSFLM. -The nutritional value of fat in muscles in BSFLM groups decreased due to the changes in the FA profile. It was concluded that the 50% or more inclusion rate of full-fat BSFLM as an alternative to SBM was too high due to its negative effects on the FA profile of meat.	(5)

For abbreviations, see the abbreviation list at the end of the chapter.
(1): Mat et al. 2022; (2): Cheng et al. 2023; (3): Oddon et al. 2021; (4): Murawska et al. 2021; (5): Daszkiewicz et al. 2022

Continuation of Table 4.

Animal	Supplementation	Result	Ref.
Broiler (Ross 308) one-day-old n=400	Full-fat BSFL C, T1, T2, T3, T4 for starter (2-10 d) %0, %2.5, %5, %7.5, %10 for grower (11-21 d) and finisher (22-42 d) %0, %5, %10, %15, %20 Exp. period: 42 days	-No effects on the muscle, total fat, total bone, muscle/body weight, and fat/body weight ratios -No effects on the broiler breast meat, drumstick, thigh, or any cut yield evaluated such as breast/body weight, drumstick/body weight, and thigh/body weight ratios, -No effects on meat pH, color parameters (L*, a*, b*, C*, and H*), or lipid oxidation, cooking loss and shear force of breast meat, -Amino acid serine concentration of breast meat in T2 was lower than C. However, aspartic acid, glutamine and lysine concentrations in T1, T2 and T3 were higher than C -An increase in the dietary level of BSFL increased decanoic acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), and isoheptadecanoic acid (C17:0), however, it resulted in a linear decrease in stearic acid (C18:0), eicosanoic acid (C20:0), and behenic acid (C22:0) in the breast meat, - The inclusion of BSFL in the diet did not affect MUFAs. - Total PUFAs decreased, but eicosapentaenoic fatty acids increased by 78% in T4. It was concluded that dietary full-fat BSFL inclusion up to 20% did not affect meat characteristics but positively increased the levels of omega-3 fatty acid EPA in meat.	(6)
Broiler (Ross 308) one-day-old n=126	Replacement of SBM with microwave-dried BSFLM C, T1, T2 C: %0 replacement T1: %25 replacement T2: %50 replacement Exp. period: 35 days	-The final LBW, ADFI and ADG in C and T1 were higher than T2. -The FCR tended to increase with the inclusion level of BSFLM. -The apparent ileal digestibility of CP in C and T1 was higher than T3, however, digestibility of DM and energy was not affected. It was concluded that 50% substitution negatively affected growth performance by malnutrition caused by low protein digestibility, however, 25% substitution showed no detrimental effects on growth performance and health in broilers.	(7)

Broiler (Cobb) one-day-old n=90	BSF prepupae meal C, T %0, %5 Exp.period: 3 weeks	-No differences between C and T for BWG, FI and FCR. -No differences between C and T for CP digestibility, length or weight of duodenum, jejunum, ileum, caecum and weights of liver, proventriculus, gizzard, bursa and spleen. It was concluded that BSF prepupae meal could be incorporated at 5% in broiler diet.	(8)
Broiler (Ross 308) one-day-old n=252	Defrosted whole BSFL C, T1, T2, T3 %0, %10, %20, %30 Exp. period: 6 weeks	-No differences among groups for slaughter weight, hot carcass weight and breast weight. -No differences among C, T1 and T2 for breast DM%. -No differences among groups for dressing, breast, legs, wings and abdominal fat percentage (% of carcass weight) -No differences among groups for breast meat quality (WHC percentage, pH, L^*, a^*, b^*, C^*, H^* values) -No differences among groups for CP content (DM%) of breast meat. -The proportion of conjugated linoleic acid in muscle and abdominal fat was highest in T3 followed by T2 and T1 compared with C. The PUFA level in abdominal fat was lower in T3 than C. It was cocnluded that whole BSFL could be included in broiler diets up to 20 without adverse effects on slaughter weight, meat quality and FA compositions, whereas, the highest inclusion altered FA composition in plasma, fat and meat.	(9)

For abbreviations, see the abbreviation list at the end of the chapter.
(6): de Souza Vilela et al. 2021a; (7): Kim et al. 2022; (8): Elangovan et al. 2022; (9): Seyedalmoosavi et al. 2022

As in every new feed or feedstuffs, there are also studies investigating the effects of BSF larvae on the yield performance and quality of laying hens. It was reported that the presence of BSF larvae meal in the diet could affect the enzymatic events in the small intestine and the formation of volatile fatty acids in the ceca, these changes positively affected the production of butyric acid in the ceca, and could have a negative effect on the enzymatic events in the ileum, therefore, a balance between negative and positive effects should be ensured in determining the optimal supplementation level of BSF larvae (Lopez et al., 2022).

Maurer et al. (2016), who investigated the effects of partially defatted dry BSF larvae meal in layer hens, reported that the use of 12 and 24% BSF larvae meal in the experimental groups, corresponding to 50% and 100% of the SBM in the diet of the control group, caused no significant difference between the groups in terms of egg production, FI, egg weight and feed efficiency. It was also observed that albumin weight tended to be lesser without any difference in yolk and shell weights, and the use of BSF larvae meal instead of 100% of SBM in the diet was a very high level (24%) and greatly increased the fecal dry matter and blackness of feces in animals. In layer hens, Bovera et al. (2018) conducted a study in which SBM was replaced by BSF larvae meal at a rate of 20, 25 and 50%, they reported that there was no significant difference between control and experimental groups in terms of egg weight, FI and FCR, and experimental groups had signifantly higher egg mass than those of control . The 25% BSF larvae group had the best rate in terms of laying percentage, and the DM, OM and CP digestibility coefficients were the lowest in the 50% BSF larvae group. A decrease was observed in serum cholesterol and triglyceride levels in both BSF larvae groups, but the albumin/globulin ratio decreased in the 50% BSF larvae group due to the decrease in serum globulin levels. As a result, in this study, it was concluded that the replacement of 25% of SBM with BSFL was more appropriate and closer to the optimal level in laying hens. Al-Qazzaz et al. (2016), who added 0%, 5% and 1% BSF larvae meal into layer hens diets with 155, 140 and 170 energy/protein ratio respectively, reported that FI, BWG, Haugh unit and hatchability were not affected by BSF larvae meal supplementation, egg appearance, texture, taste and acceptace of eggs significantly improved in 5% BSF larvae group. It was also determined that there was significant improvement in BSF larvae groups in terms of hen day egg production ([number of eggs produced on dailiy basis / number of birds available in the flock on that day] × 100) and hen house egg production ([total number of eggs produced by flock / total number of hens housed] × 100), and it was concluded that BSF larvae could be a suitable source of protein in laying hens. In a study on pullets (Mwaniki et al.,2018) in which corn-SBM-based diets were formulated with 0%, 5.0% and 7.5% BSF larvae meal, there was no significant differences between control and 7.5% BSF larvae meal group for hen-day egg production, egg weights and egg mass, however, 5.0% BSF larvae meal group had significantly lower hen-day egg production than control and other experimental group. In this study, the use of BSF larvae meal caused a significant linear decrease in FI and FCR (FI/egg mass), while an increase was detected in yolk color, shell-breaking strength and shell thickness values, but individual egg weight and haugh unit were not affected. The results of some recent studies in layer hens are presented in Table 5.

The effects of BSF larvae meal were also investigated in layer quails. Decrane et al. (2017) performed an experiment to evaluate the the effects of BSF larvae meal on live performance and egg quality in layer quails, and they added 0%, 10% and 15% BSF larvae meal into what-soybean flour based diets without altering protein and energy levels. In this experiment, no significant difference was found between groups for egg sensory characteristics, egg weights, egg surface area, albumin pH values, egg dry matter, lipid and cholesterol contents, egg SFA, PUFA n-6 and n-3 levels, egg yield and final body weight of animals. In BSF larvae meal groups, egg shape percentage, egg shell percentage, yolk color and shelf-life (due to low mg MDA/kg egg ratio) and egg MUFA levels were significantly higher than control, however, egg edible portion percentage and egg shell thickness were lower in the BSF larvae meal groups compared to the control. As a result, it was reported that the addition of BSFL larvae meal up to 15% in laying quails was appropriate. In a recent study on layer quails conducted by Harlystiarini et al. (2020), 6.57% and 13.15% BSF larvae meal was added into diet, corresponding to 50% and 100% of FM used in the diet, respectively. It was reported that there was a significant increase in egg production and egg mass in the group supplemented 13.15% BSF larvae meal, and it was concluded that the use of BSF larvae meal as an alternative to fish meal up to 13.15% in layer quails had a positive effect on egg production.

Table 5. *Results of the recent studies on laying hen performance*

Animal	Supplementation	Result	Ref.
Hens (Shaver White) 28-week old n=108	Replacement of SBM with defatted BSFLM C, T1, T2 %0, %10, %15 Exp. period: 28 to 43 weeks of age	-HDEP, FI and HU were not affected. -Egg mass decreased and FCR increased in T1 and T2. -Shell breaking strength and yolk color increased in T1 and T2. -Empty ceca weight reduced, liver weight increased and no effects on gizzard, small intestine and pancreas weight in T1 and T2. -Apparent metabolizable energy increased in T1 and T2. It was concluded that defatted BSFLM can totally replace SBM without negatively affecting HDEP. The inclusion of defatted BSFLM resulted in deeper orange yolks, improved eggshell quality, however, decreased egg weight due to poor FCR.	(1)

Hens (Julia) 178-day old n=87	Substituting FM with defatted BSFLM in maize-SBM based diet C, T1, T2 C: %3 FM T1: %1.5 FM + %1.5 BSFLM T2: %3 BSFLM Exp. period: 52 weeks	-No effect on the laying rate, FI and FCR. -LBW in T2 was higher than C, but not in T1. -No difference between groups for eggshell parameters, albumen weight, Haugh unit, yolk height and yolk color. -Yolk weight in T1 and T2 was higher than C. It was concluded that defatted BSFLM could be used as a poultry feed ingredient without any adverse effect, and substitution of FM with BSFLM could be a feasible to contribute to the laying hens performance and farming cost.	(2)
Hens (Lohmann Brown) 55-week old n=60	Replacement of SBM with BSFLM C, T1, T2, T3, T4 C: %0 replacement T1: %25 replacement T2: %50 replacement T3: %75 replacement T4: %100 replacement Exp. period: 16 weeks	-No differences among groups for average egg weight, egg albumen weight and Haugh unit. -T1 and T3 had higher average shell thicknes than C. -T2 had higher average weight of the shell than other groups, however, no differences among C, T1, T3, T4. -T1, T2 and T4 had higher relative weight of shell than C. -T2 and T4 had lower relative weight of albumen than C and T3. -T2 had higher albumen high than C, T1 and T4. -T2 had higher yolk weight than C and T3. -T4 had higher relative weight of yolk than C and T3. -T4 and T3 had higher yolk high than C and T1. Also, T3 had higher yolk high than T1. -T2 had the lowest yolk index, however, T3 had higher yolk index than C and T1. It was concluded that the replacement of SBM by BSFLM produced a significant improvement in shell thickness, shell weight, shell weight relative, albumen weight relative, albumen height, yolk weight, yolk weight relative, yolk height, yolk index, and BSFLM could be used as a successful and economical alternative in laying hens diets to improve some egg quality characteristics.	(3)

Hens (Dekalb White) 65-week old n=352	Live BSFL C, T C: Com. diet with %9.7 SBM T: SBM-free diet + 12 g live larvae per hen per day (%10 of DFI) Exp. period: 12 weeks	-T had lower FI and FCR than C. -No differece between C and T for BW, laying rate, egg weight, egg mass, breaking strenght, elasticity and HU. -Feather damage in T was lesser than C. It was concluded that replacing SBM with live BSFL had no adverse effect on production performance and egg quality, and had positive effect on feather condition in older laying hens.	(4)
Hens (Bowans White) 18-week old n=40	Live BSFL C, T1, T2, T3 %0, %10, %20, *ad libitum* Exp. period: 18-30 weeks of age	-T3 gained more weight than other groups. -No effects of larvae on egg production, egg weight, shell thickness, shell breaking strength and Haugh unit. It was concluded that ad libitum feeding of live BSF larvae had no strong effects on egg production or egg quality, but did reduce feed consumption and increased hen weight.	(5)

For abbreviations, see the abbreviation list at the end of the chapter.
(1): Mwaniki et al. 2020; (2): Zhao et al. 2022; (3): Zaki & Naji, 2022; (4): Star et al. 2022; (5): Tahamtani et al. 2022

It is noteworthy that the use of BSF larvae at a high rate in poultry feeds leads to a decrease in performance, especially in feed consumption. Considering that the birds are sensitive to the color of the feed, aside from the increase in the amount of chitin in the feed, it is possible that the BSF larvae meal, which has a darker color than the SBM, may have an effect on this (Dörper et al., 2021).

The Effects of BSF Larvae on Immune System and Antimicrobial Activity in Poultry

Immunity in poultry is an organic system that provides the animal's defense mechanism against external threats and in which many tissues, cells and molecules interact with each other. The proper functioning of this system depends on meeting the nutrient and energy needs of the immune cells (Sáenz, 2021). It is known that nutrients affect the response to diseases in poultry. The use of amino acids produced by the breakdown of body proteins by special cells responsible for synthesizing critical proteins that allow a successful immune response against the disease agent is can be cited as an example for this. Factors such as anatomical development of lymphoid tissues, mucus production, synthesis of immunologically active substances, cellular proliferation, cellular activation and movement, intracellular destruction of pathogens, modulation and regulation of immune

process can be affected by nutrition in poultry (Butcher and Miles, 2018). In order to meet the ever-changing demands in the global food market, initiatives such as genetic inter- or intra-line selection play a key role in the poultry industry to prevent vertically transmitted diseases and to increase livability by increasing resistance to diseases, however, selection based on growth characteristics and other phenotypic characteristics may adversely affect the immune status of animals. and may make them more susceptible to diseases (Swaggerty et al., 2019). In addition to many feed additives tested as immune system enhancer in poultry, there are also studies on this subject related to BSF larvae, which has attracted attention as an alternative protein source in recent years.

It has been reported that BSF larvae have immunomodulatory properties due to their chitin, lauric acid and several antimicrobial peptides. In the immune modulation, chitin and its derivatives have direct and indirect effect by creating changes in the microbial community in the animal, while lauric acid and antimicrobial peptides have an indirect effect by creating a change in the microbial community (Dörper et al., 2021). Chitin forms the main structure of the exoskeleton in insects. It was confirmed by some studies that chitin, a non-toxic and biodegradable linear polymer, had effects on innate and adaptive immune response by activating innate immune cells and stimulating cytokine and chemokine production (Edea et al., 2022). It was also reported that chitin and lauric acid prevented the penetration of harmful bacteria into the cell membrane by creating a scavenging effect on them through cationic properties (Yi et al., 2014). In a study in which 5% BSF larvae oil was added instead of soybean oil in turkeys, it was determined that the proliferation of *Enterobacteriaceae* and the level of IL-6 decreased, also, TNF-α concentration decreased when half of soybean oil replaced by BSF larvae oil, and it was reported that these effects were caused by the high amount of lauric acid in BSFL oil (Sypniewski et al., 2020).

Among the antimicrobial effects, improving innate immune reactions and selective immunoregulatory effects of antimicrobial peptides can be counted (Zyowska et al., 2011). In addition, antimicrobial peptides derived from BSF larvae are thought to have an alternative potential to antibiotics in the prophylaxis and treatment of diseases in animals, due to their antimicrobial properties and low level of resistance (Xia et al., 2021). In a study on antimicrobial peptides in BSF larvae, a total of 53 genes accepted as antimicrobial peptides and 6, 7, 26, 10 and 4 of them were identified as attacins, cecropins, defensins, diptericins and knottin-like peptides (Vogel et al., 2018). The antibacterial effect of BSF larval extract rich in antimicrobial peptides was proven in an *in vitro* study by Harlystiarini et al. (2019). In this study, six dilution levels of larval extracts (10, 20, 40, 80,

160 and 320 mg/ml) were tested against two important bacterial strains for poultry, *Salmonella sp.* and *Escherichia coli*. As a result of the study, it was determined that 320 mg/ml dilution level of BSF larval extract exhibited a strong antibacterial effect against these bacteria, and it was also stated that the obtained larval extract contained a high amount of lauric acid (49.18%), a saturated fatty acid proven as an antibacterial agent. In broilers infected experimentally with *S. gallinarum*, it was observed that the addition of 1%, 2% and 3% BSF larvae into the diet significantly increased the percentage of CD3+ and CD4+ T lymphocytes, serum lysozyme activity and spleen lymphocyte proliferation, survivability against *S. gallinarum* in the experimental groups compared to the control group, and it was suggested that BSF larvae had prophylactic properties stimulating non-specific immune responses (Lee et al., 2018). In a trial in quails, Harlystiarini et al. (2020) determined that the addition of BSF larvae meal (13.15%), which was used instead of 100% of the FM, into the diet significantly increased the antibody titer against avian influenza virus and the phagocytic activity and capacity of macrophages without risking the health status of animals, and, as a result, they reported that BSF larvae meal could be used up to 13.15% instead of FM in quails and this improved both egg production and immune response. In a broiler study conducted by El-Kaiaty et al. (2022) in which BSF larvae supplementations were at a rate of 0%, 2%, 4% and 6%, the homoral immunity antibody titers against Newcastle disease virus were significantly higher in 6% BSF larvae group on day 18, and in 2% and 4% BSF larvae group on day 28 than control, also, the supplementation of BSF larvae significantly promoted the proliferation of intestinal beneficial bacteria. Similarly, the enhancer effect of BSF larvae in the diet on the beneficial bacteria population in the digestive system has also revealed a remarkable result in a study by Ndotono et al. (2022). In this experiment performed on layer pullets fed diets in which BSF larvae meal and fish meal were replaced at certain rates, the control group was fed 100% FM + 0% BSF larvae, and the experimental groups were fed 25% BSF larvae + 75% FM, 50% BSF larvae + 50% FM, 75% BSF larvae + 25% FM, and 100% BSF larvae + 0% FM diets for 20 weeks. In the samples obtained from eight main regions of the digestive system of animals (oesophagus, crop, proventriculus, gizzard, duodenum, ileum, large intestines and ceca), there was a significant increase in beneficial bacteria such as *Lactobacillus*, *Bacteroides*, *Blautia* and *Enterococcus* depending on the increase in the BSF larvae ratio in the diet, and it was concluded that BSF larvae in layers may have a potential in terms of intestinal microbiota and health by leading to an increase in beneficial bacteria.

There is also evidence that the addition of BSFL in poultry diets does not make a significant difference in immunity. In a study in turkeys, con-

ducted by Lalev et al. (2020), the control group fed an SBM-based diet and the experimental groups supplemented with 10% defatted BSF larvae and 10% whole BSF larvae were compared, and there was no significant difference between the groups in terms of lysozyme concentrations and complement activation, and it was concluded that the addition of BSF larvae into the diet caused no significant change in the immune status of the animals. Similarly, 5% BSF prepupae meal was added into the diet in broilers by Elangovan et al. (2021), and according to the results of the cutaneous basophilic hypersensitivity test performed by using phyto hemagglutinin (PHA-P) to evaluate the cellular mediated immune response on day 21, there was no significant difference between the experimental and control groups. Considering that the differences in the substrate sources in which the larvae are grown may cause variability in the larval nutrient content, as mentioned before, it would not be wrong to think that the effects of BSF larvae on the immune status of the birds may also differ. As a matter of fact, the results of the experiment performed by Pasotto et al. (2020) on quail broilers to investigate the effects of BSF larvae grown on two different substrates, commercial chicken layer mash and 50% soaked layer mash + 50% minced fish offal, are remarkable in this context. In this experiment, the control group was fed with a quail diet, and the experimental groups were fed with a diet supplemented 10% BSF larvae grown on these two different substrates, and cellmediated immunity increased significantly in both experimental groups, and there were no significant effect on serum bactericidal activity. The humoral immune response was higher in the group fed BSF larvae reared on chicken layer mash substrate, compared to the control group, however, serum lysozyme activity was significantly higher in the group fed BSF larvae grown on fish offal substrate than control and other experimental groups. According to the results of this study, in which no significant difference was found in terms of caecal bacterial counts, the researchers reported that BSF larvae meal had immunostimulatory effects in quails, but the substrate used to rearing larvae had a strong effect on the immunostimulatory properties of larvae. The results of some recent studies on immune status in poultry are presented in Table 6.

Table 6. *Results of the recent studies on immune status in poultry*

Animal	Supplementation	Result	Ref.
Broiler (Yellow) One-day-old Exp. I: n=75 Exp. II: n=45	BSFL powder to monitör IBV infection Exp. I: C, T1, T2, T3 %0, %1, %5, %10 Exp. II: C, T %0, %10 At 10-day post hatch, chickens in the negative group and chickens in the other groups icluding the positive control group were challenged with IBV for Exp.I and II.	Exp. I: -From 5-day post challenge (dpc) infected chicks in positive C and T1 presented strong IBV symptoms (sneezing, ruffled feathers, reduced feed intake..etc). T2 and T3 presented mild symtoms. -During the experiment, the mortality in positive C, T1, T2, T3 and negative C was 13.33%, 13.33%, 6.67%, 0% and 0%, respectively, and the morbidity for T1, T2 and T3 was 92.31%, 50% and 20%, respectively. Exp. II: -At 3 dpc, IBV viral loads in trachea in T were significantly lower than positive C, however no significant differences between T and positive C for viral loads in kidney. -At 5 and 7 dpc, viral loads in tracheas and kidneys of T were significantly lower than positive C. It was concluded that the use of BSFL meal at a low percent (10%) in IBV infected young chickens enhanced immune response, reduced mortality and morbidity, activated CD8+ T lymphocytes proliferation, decreased viral loads in trachea and kidneys.	(1)
Broiler (Ross 308) one-day-old n=400	Full-fat BSFL C, T1, T2, T3, T4 for starter (2-10 d) %0, %2.5, %5, %7.5, %10 for grower (11-21d) and finisher (22-42d) %0, %5, %10, %15, %20 Exp. period: 42 days	-At 21 and 42 d, white blood cells were significantly decreased. -Blood lymphocytes were significantly reduced only at 21 d. -The subpopulations of CD3+CD8+ cytotoxic intestinal T lymphocytes decreased with increasing BSFL levels. -T4 had a 9.7-fold decrease in intestinal intraepithelial CD3+CD8+ lymphocytes compared to C. -T4 had a 5.7-fold decrease in CD3+CD4+CD8+ T lymphocytes in the intestines compared to C. -BSFL inclusion had no impact on the intestinal T helper (CD3+CD4+) intraepithelial lymphocyte populations. It was concluded that the inclusion of BSFL could potentially reduce immune response energy expenditure in broilers fed 20% BSFL for 42 d.	(2)

Turkey (Hybrid) 56-day-old n=75	Defatted and whole BSFL C, T1, T2 C: SBM based diet T1: %10 defatted BSFL T2: %10 whole BSFL Exp.period: 74 days	-No differences among C, T1 and T2 for lysozyme concentrations and the alternative pathway of complement activation. It was concluded that the immune status of turkeys from all groups did not vary significantly.	(3)
Laying quails 4-week-old n=150	BSFL meal as a replacement of FM C, T1, T2 C:%0 replacement T1: %50 replacement (%6.57 BSFL) T2: %100 replacement (%13.15 BSFL) Exp.period: 7 weeks	-No differences among groups for lymphocite, heterophils, monocyte percentage and heterophils/lymphocite ratio. -T1 and T2 had significantly higer macrophage phagocytic activity (%) and capacity than C against S.aureus non-protein A. -T3 had significantly higher antibody titer against AIV. It was concluded that the BSFL meal could be used as an alternative substitution to fish meal up to 13.15% in quails.	(4)
Laying hens 18-week-old n=200	Fresh BSFL, dried BSFL, BSFL extract C, T1, T2, T3 C: Basal ration (contained %8 FM) T1: FM reduced to %5 + %8 fresh BSFL T2: FM reduced to %5 + %8 dried BSFL T3: FM reduced to %5 + %8 BSFL extract	- The average activity of phagocytosis of peritoneal macrophages against non protein A S. aureus in T1, T2 and T3 were significantly higher than C. - The average capacity of phagocytosis of peritoneal macrophages in T1, T2 and T3 were higher than C, but not significant. -T3 had the highest value for activity and capacity. It was concluded that the combination of 5% FM + 8% BSFL extract was optimal in macrophage phagocytosis.	(5)
Layer (Hy-Line) One-day-old n=480	Full-fatted BSFL meal C, T1, T2, T3 %0, %3, %6, %9 Exp. period: 42 days	-Ileum mucosal sIgA concentration in T1, T2 and T3 was significantly higher than C, however, no differences among groups for ileum mucosal IL-2, IL-6, and TNF-α concentrations.	(6)

For abbreviations, see the abbreviation list at the end of the chapter. (1): Zhang et al. 2021; (2): de Souza Vilela et al. 2021b; (3): Lalev et al. 2020; (4): Harlystiarini et al. 2020; (5): Irawan et al. 2019; (6): Chu et al. 2020

The Effects of BSF Larvae on Antioxidant Activity in Poultry

Endogenous free radicals are formed as a result of the use of oxygen in living organisms that are dependent on oxygen for the continuation of respiration and metabolism processes, and antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase

play a role in their elimination (Altıner & Bilal, 2022). In addition to GPx, SOD, CAT, lipid peroxides (LOOH), reduced glutathione and oxidized glutathione activities, which are considered in studies investigating antioxidant status, malondialdehyde (MDA), which is a marker of endogenous oxidative damage and the end product of lipid peroxidation, is a frequently measured parameter (Calyniuk et al., 2016). In recent years, there are some evidences regarding the use of BSF larvae in poultry diets improves the antioxidant status of animals. It was reported that the addition of 5, 10 and 15% partially deflated BSF larvae meal into isonitrogenous and isoenergetic diets in broilers linearly increased the serum concentration of the GPx enzyme, which participates in the main defense mechanism against oxidative damage as a part of the antioxidant defense system in the detoxification and elimination of hydrogen peroxides, with the increase in the level of BSF larvae in diet, and the maximum effect was determined in the groups that included 10% and 15% BSF larvae (Dabbou et al., 2018). The addition of full-fat BSF larvae meal at a low level (3, 6 and 9%) into diets for 1 to 42 d in layer hens caused a linear increase in plasma GPx and total SOD activity, and a linear decrease in plasma MDA content, and it was reported that the addition of partially full-fat BSF larvae meal into the diet at the starter period improved antioxidant ability (Chu et al., 2020). In an experiment, in which dietary corn gluten meal was partially replaced by defatted BSF larvae meal in Muscovy duks, 3, 6 and 9 % BSF larvae meal was added to the experimental groups and as a result of the evaluation in terms of plasma antioxidant enzymes and oxidative metabolites in the experimental groups, no significant difference was found between the groups in terms of total antioxidant status, GPx and methylglyoxal, however, MDA and nitrotyrosine levels showed a linear decrease in parallel with the increase in the ratio of BSF larvae meal in the diet (Gariglio et al., 2019). In laying quails, the addition of 10% and 15% defatted BSFL meal into the diet showed that the values of thiobarbituric acid-reactive substances, measured to evaluate the oxidative status of egg yolk in eggs stored for 28 days, were lower than the control group, and it was determined that the group using 10% BSFL had significantly lower egg yolk MDA level compared to the control group (Zotte et al., 2019).

Conclusion

Today, valuable and expensive protein sources such as soybean meal and fish meal are widely used in poultry nutrition. It is an undoubted fact that in the future, in parallel with the increasing population, the need for animal production and therefore the protein sources required for this production will increase in order to meet the protein need. Black soldier fly larvae and meal have attracted attention as a promising alternative within

the scope of researches on finding alternative protein sources for the poultry industry, which has a great importance in meeting the protein demand in human nutrition worldwide. Studies shown that although the use of BSF larvae meal in poultry rations caused no significant differences in performance parameters such as live weight, body weight gain, feed efficiency, carcass yield, egg and meat yield, it could be used as partially replacement of protein feeds such as soybean meal and fish meal. Even, there are also evidences in some studies that this larval meal can be used completely instead of soybean meal and fishmeal. This is a remarkable situation in terms of the retrenchment of these expensive protein feeds and feed costs. In addition, it has been observed in many studies that BSF larvae meal contributes to the improvement of immunity indirectly by preventing the growth of harmful microorganisms with antimicrobial effects and creating positive effects on the proliferation of beneficial microorganisms, as well as its immunomodulatory effects. Another positive result is the evidences that the use of BSF larvae meal in the poultry diet has an antioxidant effect by activating some antioxidant enzymes and reducing especially the MDA level. Considering the extra advantages such as being easy to produce and being able to be used as an effective weapon in waste management, more and detailed studies on BSF larvae will be more enlightening on the usability of such a nutrient-rich source as an alternative feed ingredient in animal feeding.

Abbreviation List

ADFI	:	Average daily feed intake	GPx	:	Glutathione peroxidase
ADG	:	Average daily gain	HDEP	:	Hen-day egg production
AIV	:	Avian influenza virus	HU	:	Haugh unit
BSF	:	Black soldier fly	IBV	:	Avian infectious bronchitis virus
BSFL	:	Black soldier fly larvae	IL	:	Interleukin
BSFLM	:	Black soldier fly larvae meal	LBW	:	Live body weight
BW	:	Body weight	LOOH	:	Lipid peroxidase
BWG	:	Body weight gain	MDA	:	Malondialdehyde
C	:	Control group	MUFA	:	Monounsaturated fatty acid
CP	:	Crude protein	n	:	Total number of animals
DM	:	Dry matter	PUFA	:	Polyunsaturated fatty acid
EAA	:	Essential amino acid	SBM	:	Soybean meal
FA	:	Fatty acid	SFA	:	Saturated fatty acid
FCR	:	Feed conversion ratio	SOD	:	Superoxide dismutase
FI	:	Feed intake	T	:	Treatment (experimental) group
FM	:	Fishmeal	TNF	:	Tumor necrosis factor

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CHAPTER 3

CANCER IMMUNOTHERAPY STRATEGIES: TUNING THE IMMUNE SYSTEM AGAINST CANCER CELLS

Rümeysa DEMİR[1], Hüseyin Saygın PORTAKAL[2]

1 Izmir University Economics, Genetics and Bioengineering ORCID ID: 0000-0003-1132-2032
2 Research Assistant, Izmir University Economics, Genetics and Bioengineering ORCID ID: 0000-0002-3582-4152.

1. Introduction

The cancer causing the worldwide deaths for last decades is characterized by accumulation of mutations and the loosing of regular metabolic functions by the cancerous cells. To date many therapy approaches such as chemotherapy based on to design chemical therapeutic compounds that might target biomarkers of cancer (Anand et al., 2022), radiotherapy based on to kill the cancer cells by using high dosage of radiation (Baskar, Lee, Yeo, & Yeoh, 2012), gene therapy based on manipulation of gene editing tools such as CRISPR/Cas9 (Portakal, 2023), RNAi (Chalbatani, Grijalvo, Eritja, Logsdon, & Memari, 2019) etc. have been developed against various cancer types. However, many strategies of cancer cells such as creating tumor tissues through over growing and accumulation of cancerous cells, metastasis, enhancing the mutation burden limits the delivery of the therapeutic agents and their efficiencies (Chakraborty & Rahman, 2012). As such, to develop novel therapy approaches against cancer disease are the main topics of health sciences still.

While the immune system is body defense system in all species, it's one of the most complex body system due to its working principle is based on various interactions created through surface receptors or secreted special biomolecules by specific immune cells. Mammalian immune system is composed of two distinct immunities which are innate immunity providing first and second lines of defense and adaptive immunity providing third line of defense via its memory and specificity (Marshall, Warrington, Watson, & Kim, 2018). Apart from that immune system has developed powerful strategies in the combat with foreign infecting organisms, it has also quite effective and complicated mechanism in order to distinguish and destroy the genetically disordered cells such as cancer cells (J. Zhang, Han, Zhang, & Zhang, 2014). In this scope Cancer Immunity Cycle is promoted by immune system with following steps; i) the tumor neoantigen releasing by oncogenesis and recognition of them by dendritic cells (DCs), ii) processing and presenting the neoantigens to T cells via major histocompatibility complex I and II (MHCI and MCHII), iii) priming and activation of effector cytotoxic T lymphocytes (CTLs) in lymph nodes, iv) migration and infiltration of CTLs to tumor microenvironment (TME), v) degradation of cancer cells with secretion of interferon-γ (IFN- γ) and cell mediated response of CTLs (D. S. Chen & Mellman, 2013). Degradation of cancer cells causes to releasing of neoantigens to TME and cancer immunity cycle is repeated.

Although the existence of cancer immunity cycle, cancer cells have found many strategies in order to escape from immune response (Tang, Ning, Yang, Mo, & Tang, 2020). For instance the T regulatory cells (Tregs) concentration (Grover, Goel, & Greene, 2021), inflammatory conditions

of TME (Tan et al., 2021), the obstacles that are encountered during migration of DCs and T cells (Lucarini et al., 2021) are main anatomical parameters describing the effect of cancer immunity cycle. In addition, the parameters like regulation of TME aiming to inhibit CTLs infiltration (Klemm & Joyce, 2015), metastasis to other body portions (Fares, Fares, Khachfe, Salhab, & Fares, 2020), to induce inhibitory cytokines expression (Hrdý et al., 2020), the reduced expression level of MHCI (Cornel, Mimpen, & Nierkens, 2020) are the several strategies of cancer cells so that to escape from cancer immunity cycle. Furthermore, there are some immune checkpoint proteins such as CTLA4 or PDL1 that might interact with B7.1 receptor of T cells in order to stop immune response (Buchbinder & Desai, 2016). Another most significant escape method of cancer cells is overexpression of such those immune checkpoint surface proteins. The manipulation of these information has created a recent area with the name of cancer immune therapy in our era. As such, designing and producing of immune checkpoint inhibitors (Robert, 2020), targeted monoclonal antibodies (mAbs) (Kumar, Thangavel, Becker, & Sadayappan, 2021), to develop adoptive Car-T cell therapy (Sterner & Sterner, 2021a), and cancer vaccines (Liu et al., 2022) are main research areas in health sciences. The scientists aim to re-educate the immune system in order to overcome anatomical barriers and cancer strategies that have been developed to escape from cancer immunity cycle. Many great advancements have been recorded in this area for last decades, and the cancer immunity cycles as well as the working principles of cancer immunotherapy approaches are introduced and discussed in this publishing.

2. Cancer Immunity Cycle

Immune system is one of the most complex body systems of mammalian species and it provides defense mechanisms to foreign dangerous materials such as infecting organisms or products of genetically disordered host cells. While immune system acts with two main strategies which are innate immunity and adaptive immunity, many distinct cell types have special objectives in the combat with foreign particles (Iwasaki & Medzhitov, 2015). Due to the fact that tumor tissue might cause to death through creating huge damages in the body, cancerous cells are one of the main targets of the immune system. In particular, an effective strategy of immune system including the connections between innate and adaptive immunities is promoted against cancer progression (Pardoll, 2015). This immune response of the body containing repeated stepwise series is named as Cancer Immunity Cycle and is one of the major topics of many researches in health sciences carried out for many years (D. S. Chen & Mellman, 2013).

The working mechanism of cancer immunity cycle is illustrated in Figure 1. The first step of the cycle initiates with the releasing of tumor neoantigens produced during oncogenesis, and recognition of them dendritic cells (DCs) which are one of the antigen presenting cells acting in immune system (Xie et al., 2023). Once the recognition, neoantigens are processed and presented to T cells through major histocompatibility complexes (MHCI and MHCII) of DCs (Jiang et al., 2019). This step leads to prime and activation of effector cytotoxic T lymphocytes (CTLs) in secondary lymphoid organs (Busselaar, Tian, van Eenennaam, & Borst, 2020). Following, activated effector CTLs migrate to tumor carrying tissue via blood vessels and infiltrate to tumor microenvironment (TME) (Boissonnas, Fetler, Zeelenberg, Hugues, & Amigorena, 2007). Eventually, cancer cells are degraded by the interferon-γ (IFN- γ) secretion and cell mediated response of CTLs after creation of the interactions between T cell receptor (TCR) and MHCI of cancer cells (S. C. Chen, Wu, Wang, & Kuo, 2020). The degradation of cancer cells cause to release of neoantigens to environment and cancer immunity cycle is initiated to repetition. Furthermore many innate immunity cells such as granulocytes and pro-inflammatory cytokines and chemokines might boost the immune response against cancerous cells (Lan, Chen, & Wei, 2021). In addition, while many cytokines such as tumor necrosis factor-α (TNF- α), interleukin 1 (IL1), IFN- α, IL-2, IL-2 (Dinarello, 2006) etc. and protein interactions such as CD40L/CD40, CD28/B7.1, CD137/CD137L, CD27/CD70, LFA1/ICAM1 (Jeong & Park, 2020) play stimulatory roles, several cytokines such as IL-10, IL-4, IL-13 (Briukhovetska et al., 2021) etc. and protein interactions such as PDL-1/PD1, CTLA4/B7.1, PDL1/B7.1 (Turnis, Andrews, & Vignali, 2015) play inhibitory roles in each cycle of cancer immunity cycle.

However, the effect of cancer immunity cycle might be interrupted with several parameters such as high content of T regulatory cells (Tregs) (Grover et al., 2021), inflammatory conditions of TME (Tan et al., 2021), migration of DCs to lymph nodes (Feng et al., 2021) etc. In addition, cancer cells have several strategies in order to escape from cancer immunity cycle. For instance, cancer cells might regulate the TME in order to inhibit CTL infiltration (Labani-Motlagh, Ashja-Mahdavi, & Loskog, 2020), metastasis to other parts of the body (Seyfried & Huysentruyt, 2013), to induce the overexpression of inhibitory cytokines (Yago, 2020), to reduce expression level of MHCI (Dhatchinamoorthy, Colbert, & Rock, 2021), overexpress the immune checkpoint modulators such as CTLA4 and PDL1 (H. Zhang et al., 2021). As such, cancer cells might escape from degradation by cancer immunity cycle and survive in host body. Today, various immunotherapy approaches such as producing of immune checkpoint inhibitors, Car-T adoptive cell therapy, cancer vaccines, and producing

of targeted monoclonal antibodies (mAbs) aiming to re-educate immune system and to overcome escaping strategies of cancer cells have been developed by great efforts of the scientists studying in the health sciences (Esfahani et al., 2020).

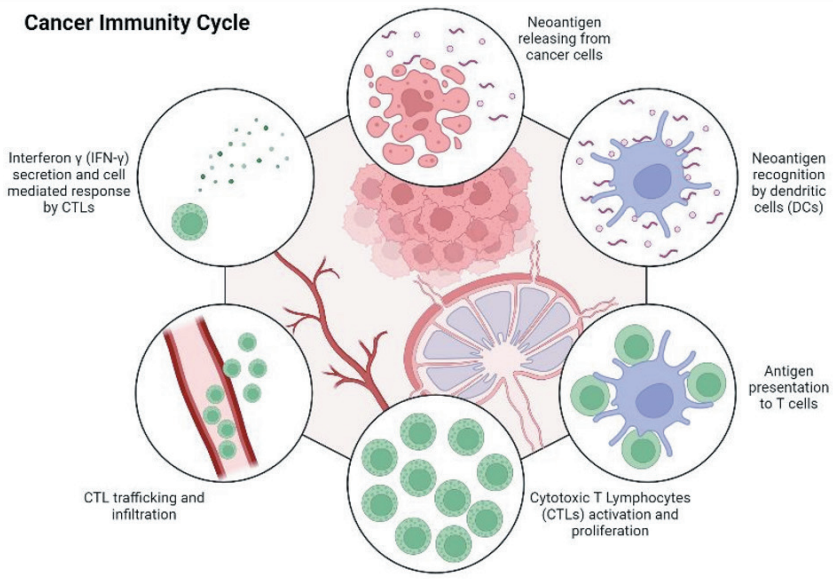

Figure 1. The steps of cancer immunity cycle.

3. Cancer Immunotherapy Strategies

3.1. Cancer Vaccines

Vaccination is the most powerful technique that is carried out for prevention against various diseases from history of mankind. Considering cancer immunity cycle, many vaccination strategies in order to re-educate and boost immune system against cancer cells have been developed for many years. While the cancer neoantigens are classified into two subclasses which are tumor specific antigens (TSA) (Apavaloaei, Hardy, Thibault, & Perreault, 2020), and tumor associated antigens (TAA) (Li et al., 2021), cancer vaccination is based on introduce the cancer neoantigens to antigen presenting cells (APC) in order to trigger CTL activation (Saxena, van der Burg, Melief, & Bhardwaj, 2021). Fundamentally, due that they are produced by cancer cells specifically, delivery of TSAs provides higher efficiency to vaccination approach comparing to delivery of TAA (Zhao et al., 2021). Considering the neoantigen and adjuvant delivery strategies, there are totally four cancer vaccine approaches which are protein or peptide vaccines (Neek, Kim, & Wang, 2019), cancer cell lysate vaccines (Kawa-

hara & Takaku, 2015), DNA or RNA vaccines (Yang, Jeang, Yang, Wu, & Hung, 2014), and viral or bacterial vector based vaccines (Guo et al., 2019) (Figure 2). Furthermore, since that DCs are main APC within cancer immunity cycle, they are the major target for neoantigen delivery in cancer vaccination methods.

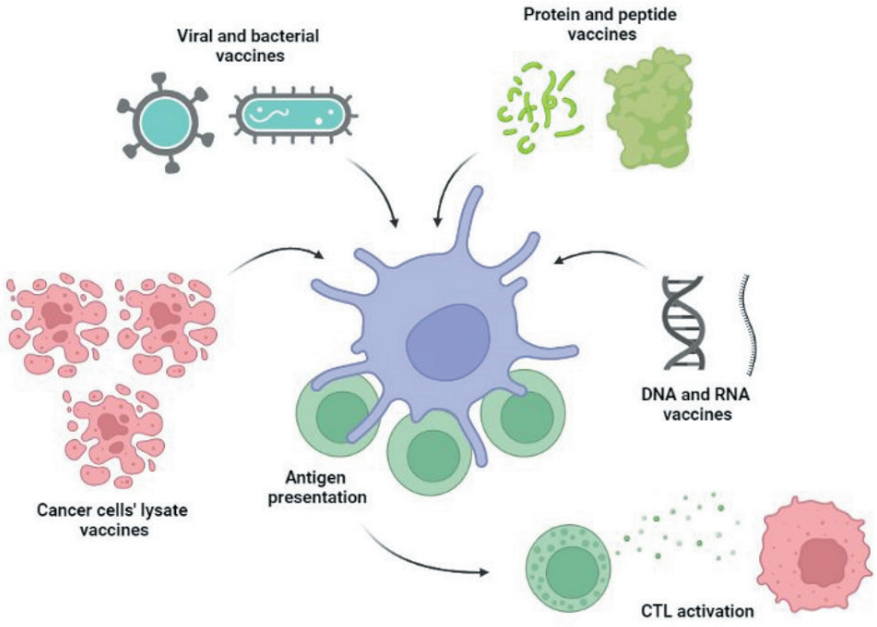

Figure 2. Cancer vaccination approaches including i) cancer cells' lysate vaccines, ii) viral and bacterial vaccines, iii) protein and peptide vaccines, and iv) DNA and RNA vaccines.

Proteins or peptide vaccines is mainly target the delivery of neoantigens to DCs in protein chemistry (M. J. Lin et al., 2022). As such, neoantigens are produced synthetically and transfected to DCs so that to be processed and presented to T cells (Stephens, Burgess-Brown, & Jiang, 2021). Neoantigen selection in protein or peptide vaccines might be carried out with two techniques which are direct immunology and reverse immunology (Rollenhagen, Sörensen, Rizos, Hurvitz, & Bumann, 2004). While reverse immunology technique is based on the prediction of epitope region of antigens (Rappuoli, Bottomley, D'Oro, Finco, & De Gregorio, 2016), direct immunology antigen selection is promoted by investigation of patient derived CTLs to analyze recognizable specific epitope regions (Davis, Tato, & Furman, 2017). However, protein and peptide vaccines might have various disadvantages such as that single epitope delivery might cause weak

immunity (Tay et al., 2021), antigen escape mechanism of cancer cells may lead to reduced expression of delivered neoantigen (Majzner & Mackall, 2018), and they might cause strong CD4 T cells response instead of CD8 T cells activation (Bouzid, Peppelenbosch, & Buschow, 2020). Nonetheless, few advantages such as cost effective production and storage make protein and peptide vaccines quite effective in cancer vaccination field.

Considering the central dogma phenomena DNA or mRNA molecules might be used for protein expression. As such, DNA and mRNA vaccines that might introduce the target neoantigen to DCs are one of the most promising vaccination methods (McNamara, Nair, & Holl, 2015). The cancer neoantigens might be transcribed from DNA molecules and translated from mRNA molecules that are containing the related gene encoded sequence within nucleus and cytoplasm, respectively (Jahanafrooz et al., 2020). Naturally produced neoantigens are processed, loaded to MCH derivatives and presented to T cells to activate cancer immunity cycle. Despite the advantages of nucleic acid vaccines such as easy production, low cost, convenient storage conditions, long term stability and durability, and high immune response (Qin et al., 2021), many biological barriers such as undesired electrostatic interactions between negatively charged nucleic acids and negatively charged cell membrane, endo and exonuclease enzymes, low pH conditions of endosome vesicles limit the transfection process of nucleic acid vaccines to target cells (Kulkarni et al., 2021). However, recently developed novel methods such as chemical modifications, nano-level viral or non-viral carrier vectors, and xeno nucleic acids (XNAs) are able to overcome such those limitations (Gewirtz, Sokol, & Ratajczak, 1998).

Since that TSAs are produced by cancer cells specifically and TAAs are overexpressed, neoantigens might be delivered through delivery of cancer cells' ingredients. Once to degrade cancer cells, their lysates are used as vaccine and in this approach lysates' neoantigens are introduced to DCs so that to initiate cancer immunity cycle (Malhotra & Mehnert, 2021). While these lysates are sourced from both cells from patient host and modified cell lines, higher specificity of patient derived cell lysates are considered as one of the novel personal medicine approach (Salewski et al., 2020). However, modified cell lines that are lysed for vaccination might be also functionalized in order to produce various adjuvant molecules such as Bacille Calmette Guerin (BCG) or GM-CSF. That is patient derived cell lysates provide specificity and modified cell lines' lysates might boost the response of cancer immunity cycle (Rojas-Sepúlveda et al., 2018). Furthermore, cancer cell lysate vaccines have significant advantages such as undiscovered neoantigens might be introduced to DCs, they might be presented to T cells without MHC restriction, cell lysates might induce both innate and adaptive immunity components (Ogino et al., 2022).

Final cancer vaccination approach is based on the usage of bacterial or viral vectors (Igarashi & Sasada, 2020). Due to the fact that the delivery of only TSAs or TAAs is not enough to achieve sufficient immune response, various adjuvant molecules may be required to boost immunity (Awate, Babiuk, & Mutwiri, 2013). As such, viral or bacterial vectors are able to be recognized as foreign by host immune system and they might induce stronger immune response. Today, viral or bacterial vectors are used to encapsulate neoantigens as well as adjuvants for delivery to DCs, and through the activation of inflammatory response these vectors boost cancer immunity cycle (Eisenberger, Elliott, & Kaufman, 2006). However, the vector selection in this vaccination should be carried out by taking into account that each vector might induce distinct types of immune response and trigger the activation of subtypes of T cells such as Th1, Th2, Th3, and Tregs (Dailey, Crosby, & Hartman, 2022).

3.2. CAR-T Cell Therapy

Chimeric antigen receptor (CAR)-T cell therapy is one of the most promising immunotherapy approaches targeting to re-educate T cells in order to attack cancer cells. Fundamentally, Car-T cell therapy is an *ex-vivo* technique since that it's based on isolation of host T-cells, cloning of CAR proteins, cultivation of CAR expressing T cells to achieve sufficient amount, and re-injection of CAR expressing T cells to the patient (Figure 3) (Huang et al., 2020). While CAR proteins are produced as special surface receptors, they provide enhanced immune response against cancer cells due to its high binding affinity and MHC independent recognition of cancer neoantigens (Alnefaie et al., 2022).

The effectiveness of CAR-T cell therapy is described by CAR proteins' structures which are designed with engineering techniques. CAR structures are composed of mainly four connecting domains which are; i) extracellular domain, ii) hinge region, iii) transmembrane domain, and iv) intracellular domain (Sterner & Sterner, 2021b). Neoantigens of cancer cells are recognized by antigen binding region of CARs' extracellular domains. This antigen binding domain is designed through deriving monoclonal antibodies' (mAb) variable heavy and light (V_H and V_L) chains. These chains are connected to one another through flexible linker to achieve single chain variable fragment (scFv) structure. While intracellular neoangitens of cancer cells are processed and presented by DCs to T cells, epitope regions of extracellular neoantigens are recognized by scFV portion of CAR in order to activate T cells against cancer cells. The affinity and specificity of antigen binding domain of CAR is the most significant parameter describing the efficiency of therapy (Chailyan, Marcatili, & Tramontano, 2011; G. Zhang et al., 2014). In addition, antigen binding domain of CAR

is connected to transmembrane domain via hinge region that is providing flexibility to protein structure. The length of hinge region is the main parameter effecting the stability and flexibility of CAR structures (Hudecek et al., 2015; Jensen & Riddell, 2015). As a surface receptor CAR proteins are anchored to cell membrane through transmembrane domain designed as including hydrophobic amino acid residues. In particular, transmembrane domain could affect the expression level of CAR proteins and they are generally derived from CD8α, CD28, and CD4 proteins (Alabanza et al., 2017; Bridgeman et al., 2010; Guedan et al., 2018). Finally, once the recognition of neoantigens by extracellular domain, T cells are activated through the cascades which are initiated by intracellular domain of CAR proteins. This activation leads to IL2 secretion and proliferation of T cells in order to provide cell mediated response against cancerous tissue (Graham, Hewitson, Pagliuca, & Benjamin, 2018).

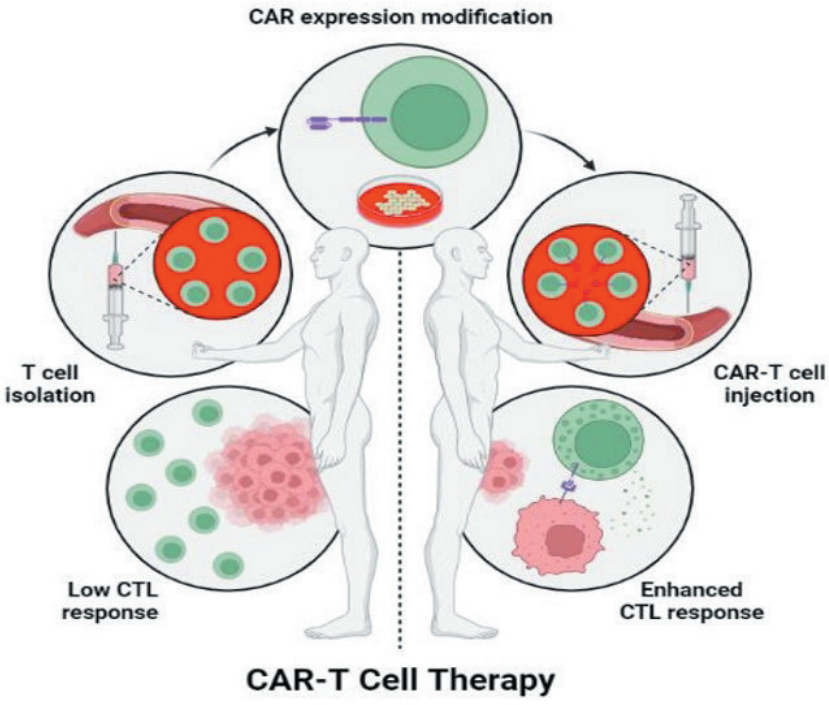

Figure 3. The working principles of CAR-T cell therapy.

Despite the effectiveness of CAR-T cell therapy, there are some limitations affecting to promote this promising techniques (Sterner & Sterner, 2021a). Antigen escape mechanisms of cancer cells is the most significant limitation that is observed in CAR-T cell therapy. In order to escape from immune response, cancer cells reduce the expression level of target antigen

that is recognized by antigen binding domain of CAR and they might express distinct neoantigens. This phenomena causes to reduce efficiency of CAR-T cell therapy (Majzner & Mackall, 2018; Maude, Teachey, Porter, & Grupp, 2015). Besides, since that cancer cells are derived from body cells, somatic cells might also express the target antigens with few level. The recognition of these antigens expressed on the surface may lead to degradation of somatic cells and this condition may cause the toxicity in the treatment. Due to this fact, antigen selection is one of the most crucial parameter for CAR design (Du et al., 2019; Steentoft et al., 2018). In addition, anatomical barriers that are observed during cancer immunity cycle also may affect the efficiency of CAR-T cell therapy. For instance, immunosuppressive conditions of TME, Treg content, interactions with immune checkpoint proteins such as CTLA4 and PD1 limit the trafficking, infiltration, and activity of CAR expressing T cells (Quail & Joyce, 2013; Whiteside, 2008). Many strategies in order to overcome these limitations have been developed by the scientists of health sciences, and these strategies are summarized in Table 1.

Table 1. The encountered limitations and developed strategies to overcome in CAR-T cell therapy.

Limitations	Developed Strategies	Reference
Antigen Escape	*Distinct antigens targeting*	*(Q. Lin, Zhao, Song, & Liu, 2019)*
CAR-T Cell Toxicity	*CAR design for TSAs*	*(Salter et al., 2018)*
TME's Suppressive Conditions	*Combinatory therapies*	*(Chong et al., 2017)*
Trafficking and Infiltration	*Localized delivery*	*(Adusumilli et al., 2014)*

3.3. Immune Checkpoint Inhibitors (ICI)

Immune checkpoints interactions are created between various surface receptors of both somatic cells and immune cells in order to prevent aggressive and autoimmune response (Dine, Gordon, Shames, Kasler, & Barton-Burke, 2017). As an escape mechanism, cancer cells might manipulate immune checkpoint interactions and they could be survive under T cell recognition. Immune checkpoint inhibitors (ICIs) are recently produced drugs that are able to block immune checkpoint interactions through binding to CTLA-4, PD-1 or its ligand PD-L1. As such, the blocking of these interactions leads to resume of immune response against cancerous cells. ICIs have substantially improved the treatment approaches for prognosis of various types of advanced cancer (Haanen & Robert, 2015).

Although CTLA-4 and PD-1 molecules were originally discovered as molecules playing a role in T cell activation or apoptosis, subsequent preclinical research showed their important role in the maintenance of peripheral immune tolerance (Kalinski & Basse, 2019). Since that these receptor proteins are manipulated by cancer cells as an escape mechanism, monoclonal antibody-based immune checkpoint inhibitors (ICIs) are recently synthesized and their quite activities have been proven with sufficient clinical results (Dine et al., 2017). Anti-CTLA-4 and Anti-PD-1 mAbs which are ipilimumab as well as nivolumab and pembrolizumab, respectively, have been approved by FDA and they affect the overall survival rates in melanoma patients (He & Xu, 2020).

3.3.1. PD-1/PD-L1 Inhibitors

The immunosuppressive protein programmed death protein 1 (PD-1) is expressed over T cells' surfaces and has significant role in the immune suppressing in order to protect host body cells. This phenomena is manipulated by cancer cells so that to create an immune escape mechanism by overexpressing PD-1's interacting ligand which is programmed cell death ligand 1 (PD-L1). As such, the interaction created between PD-1 and PD-L1 initiates a cascade stopping the activity and proliferation of T cells. Therefore, this cascade might lead to tumor evasion, and many therapy approaches might fail against this phenomena. Understanding the mechanism within PD-1/PD-L1 associated cancer evasion is quite crucial for creating combinatory immunotherapy. Today, targeting this interactions is one of the most significant immunotherapy candidate since that it has exhibited quite efficiency (Thibult et al., 2013).

PD-1 is a checkpoint protein member of the CD28 family. Mainly, it refers to a class of suppressor T-cell receptors whose expression level is increased during antigen and cytokine stimulations (Kinter et al., 2008; Kulpa et al., 2013). PD1 is expressed by also B cells, monocytes, and dendritic cells (DCs) in order to modulate their immune activity (Keir, Butte, Freeman, & Sharpe, 2008). PD-L1 is a member of B7 ligand family and it's a type 1 transmembrane glycoprotein. The expression of PD-L1 is observed on the surface of somatic cells (Zou, Wolchok, & Chen, 2016). The interactions between PD-L1 on somatic cells and PD-1 on T cells may lead to signal inhibiting autoimmunity. The interaction between PD-1 and PD-L1 regulates the activity and proliferation of T cells. However, this interaction is manipulated by cancer cells to escape from cancer immunity cycle. In principle, PD-1 recognized cancer cells upregulate PD-L1 expression so that they might survive under T cell recognition. (X. S. Li, Li, Li, & Jiang, 2020; Y. Li et al., 2015; Topalian, Taube, Anders, & Pardoll, 2016; Tremblay-Lemay, Rastgoo, & Chang, 2018). Furthermore, the IFN-γ secretion

via CTL response also causes the upregulation of PD-L1 in cancer cells. PD-1/PD-L1 interactions may also suppresses infiltration of CD4+/CD8+ T lymphocytes (CD4+/CD8+ TILs) and causes a reduction in various cytokine production such as tumor necrosis factor (TNF), IFN- γ, and Interleukin-2 (IL-2). Anti-tumor T cell immunological suppression is unblocked by PD1/PDL1 inhibitors, resulting in T cell proliferation and penetration into the TME and induction of an anti-tumor response (Kuzume et al., 2020). Existing anti-PD1/PDL1 therapy prevents the interaction of PD1 and PDL1, successfully activating depleted immune cells and inducing an anti-tumor immune response (Seidel et al., 2018).

Numerous preclinical studies have demonstrated that the restoring T cell function by blocking PD-1 or PD-L1 through mAbs is quite promising in treatment of various cancers including breast cancer, melanoma, glioblastoma, lung cancer, kidney cancer, colorectal cancer, and ovarian cancer (Cubas et al., 2018). To date, many Anti-PD-1 mAbs such as nivolumab, pembrolizumab, cemiplimab, as well as Anti-PD-L1 mAbs such as atezolizumab, avelumab, and durvalumab have been developed with sufficient clinical advancements and they have been approved by FDA as the drugs that might be used in cancer immunotherapy.

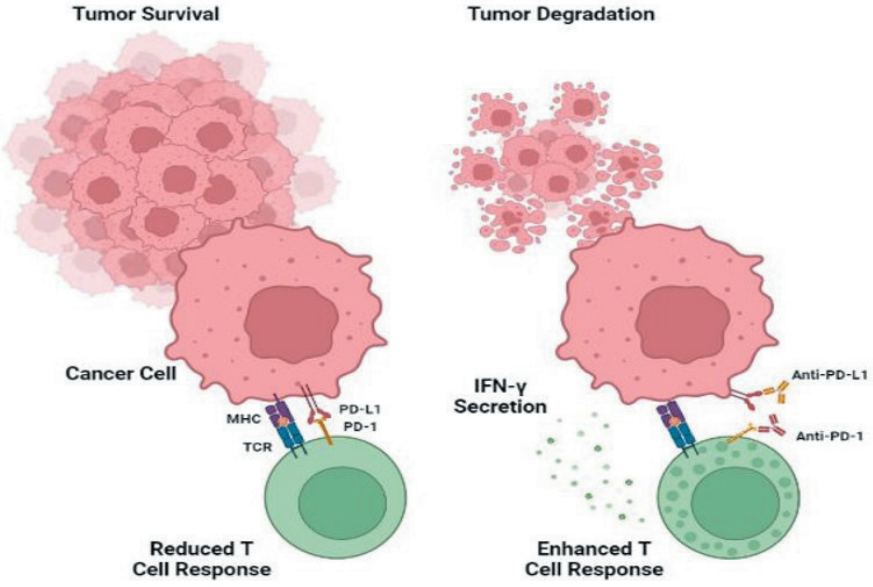

Figure 4. Anti-PD-1 and Anti-PD-L1 therapies in order to inhibit cancer escape from CTL response.

3.3.2. CTLA-4 Inhibitors

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is another immune checkpoint regulating T cell activation. The CTL response requires the naive T lymphocytes activation through two main signals which are TCR interactions with antigen loaded MHCs and CD28 interactions with B7-1 (CD80) or B7-2 (CD86). While CTLA-4 is expressed over T cells, it may block the CD28 signal through its high affinity to B7-1 and B7-2 receptors (Qureshi et al., 2011; Walker & Sansom, 2011). The interaction between CTLA-4 and B7-1 or B7-2 prevents the TCR downstream phosphorylation cascade and thereby inducing T-cell tolerance (Hammers et al., 2017; Marable et al., 2021; Massard et al., 2016). Moreover, tumor-infiltrating regulatory T cells (Tregs) express CTLA4 and use it to create an immunosuppressive environment in TME (Friedline et al., 2009; Walker & Sansom, 2011). Just as in PD-1/PD-L1 case, inhibiting of CTLA-4 with mAbs is quite promising in the treatment of several cancers. For instance, ipilimumab is one of the most significant imunnotherapeutic candidate in the treatment of human melanoma patients (Eggermont et al., 2016; McDermott, Haanen, Chen, Lorigan, & O'Day, 2013). Ipilimumab's inhibition of CTLA4-B7 interaction allows for unrestricted CD28-mediated positive stimulation and activation of cytotoxic T-cell responses (Peggs, Quezada, Chambers, Korman, & Allison, 2009; Ramagopal et al., 2017). Although antibodies that inhibit CTLA-4 interactions with its ligands, such as ipilimumab, have been shown to have anticancer activity and therapeutic advantages (Ramagopal et al., 2017), it is always preferable to have bioavailable and less expensive alternatives in the form of small molecules or peptides. In circumstances where no acceptable small-molecule binding pockets can be quickly discovered at the ligand-binding interface, peptide medicines can provide a potential alternatives to block target interactions (Sobhani et al., 2021). Since that peptides have smaller size then mAbs, the production of peptide based drugs are assessed as low cost and efficient since that they are able to penetrate target tissue easily (Sobhani et al., 2021).

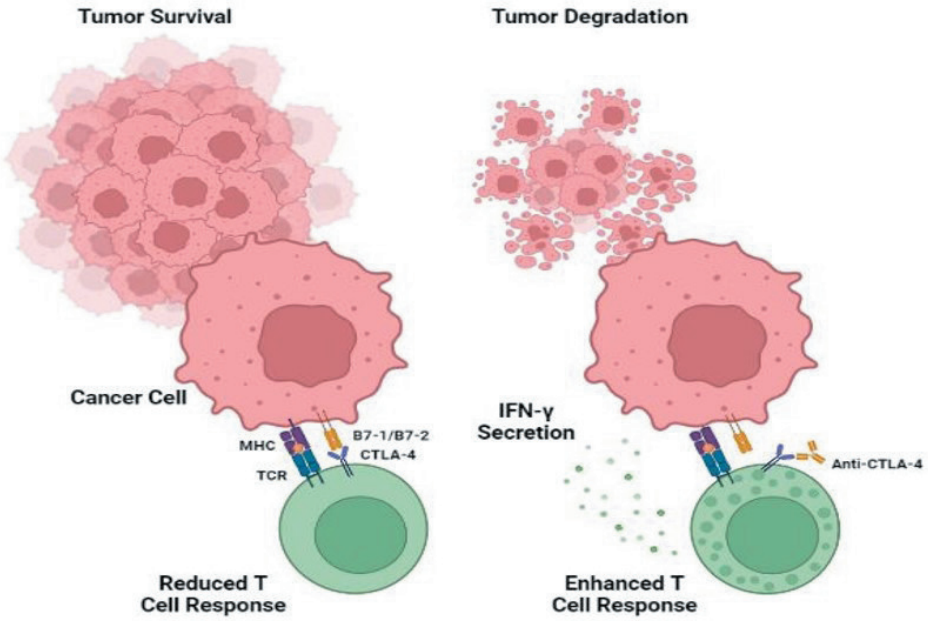

Figure 5. Anti-CTLA-4 therapy in order to inhibit cancer escape from CTL response.

3.4. Monoclonal Antibody (mAb) Therapies

Antibodies are naturally produced biomolecules by immune cells that identify a particular target. Foreign small sized particles (antigens) are recognized by antibodies that are produced by B-lymphocytes and an immune response is developed. The recognition regions of the antigens are named as epitopes. A monoclonal antibody (mAb) is an antibody that is generated against a specific epitope rather than a distinct epitope regions of various antigens (Kohler, 2000). This phenomena makes mAbs as biomolecules with high specificity. In the context of cancer treatment, mAbs can recognize neoantigens or other surface proteins expressed by cancer cells in order to initiate a signal inducing apoptosis.

The usage of mAb based immunotherapeutics is innovative therapy and it's mainly based on the antigen recognition by Fv (variable fragment) region and induce the host immunity through Fc (constant fragment) region (Harris & Drake, 2013). In clinical practice, mAbs are the most extensively used and approved cancer immunotherapy method. mAbs are most typically used to treat breast, colon, lymphomas, and other types of cancers (Sathyanarayanan & Neelapu, 2015).

Several mAbs utilized in cancer immunotherapy target and bind to neoantigens on the cancer cells' surface to inhibit certain downstream sig-

naling cascades and cell growth (Papaioannou, Beniata, Vitsos, Tsitsilonis, & Samara, 2016). Immunotherapeutic mAbs are classified into two subclasses based on the functionalization with chemical compounds or radioactive substances. In addition several non-conjugated mAbs such as alemtuzumab and transtuzumab are able to induce apoptosis for cancer cells. However it's revealed that chemotherapeutic or radioactive particles conjugated mAbs have higher efficiency to inhibit cancer growth. For instance, gemtuzumab ozogamicin is an drug-conjugated mAb, and Ibritumomab thiuxetan is a radioactive conjugated mAb (Karlitepe, Ozalp, & Avci, 2015; Oldham & Dillman, 2008; Sathyanarayanan & Neelapu, 2015). In addition, novel approaches that might enhance the specificity of mAbs such as production of bispecific antibodies (bsAb) that might target several antigens, for instance Blinatumomab, has been developed with quite effectiveness (Özlük et al., 2017).

4. Conclusion

To date, various therapeutic approaches such as chemotherapy using the chemical drug ligands targeting biomarkers sourced from cancer cells, radiotherapy using radiation that might destroy cancerous tissue, and gene therapy using therapeutic nucleic acid agents have been developed against cancer diseases. However lots of parameters could limit the efficiencies of such those approaches, and novel therapeutic approaches are required to be developed with quite effectiveness and specificity. Immune system has quite significant strategy to destroy cancer cells named as cancer immunity cycle and it's based on to work of both innate and adaptive immunity. As such, cancer immunity cycle is including the cycles; i) the releasing of neoantigens and capturing of them by DCs, ii) processing and presentation of neoantigens to T cells, iii) priming of effector CTLs, iv) trafficking and infiltration of CTLs to TME, v) to destroy of cancer cells via interferon-γ (IFN- γ) secretion and cell mediated response of CTLs. However, several anatomical limitations and escape mechanisms of cancer cells might cause to reduce effect of cancer immunity cycle. A novel therapy approach, cancer immunotherapy, has born last decades and it is aiming to re-educate the immune system so that it could complete the cycle with sufficient efficiency. In this scope many techniques such as cancer vaccines, immune checkpoint inhibitors, adoptive Car-T cell therapy, and targeted monoclonal antibodies (mAbs) have been developed in health sciences. The cancer immunity cycle as well as the working principles of developed techniques underlying cancer immunotherapy approach have been reported and discussed in this chapter.

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CHAPTER 4

GLASS IONOMER CEMENT: PAST AND FUTURE

Mediha BÜYÜKGÖZE DINDAR[1]

1 Dr. Öğretim Üyesi, Trakya Üniversitesi Sağlık Hizmetleri Meslek Yüksekokulu, Ağız ve Diş Sağlığı Programı, ORCID ID: 0000-0003-3794-4366

Conventional glass ionomer cement (GIC), formed through the acid/base reaction between calcium (or strontium) aluminosilicate and polyalkenoic acid, was developed in the mid-1970s to provide an alternative solution to the commonly used amalgam restorations in the posterior region (Sidhu & Nicholson, 2016). Unfortunately, both the clinical longevity and overall quality of these chemically curing restorative materials ended up to be only lower and not advisable for posterior use. Although the various material improvements made promise to provide exceptional properties such as chemical adhesion, simple application, moisture tolerance, along with bioactive properties (anti-microbial and anti-cariogenic effects due to a significant amount of fluoride release), applicators still deal with significant disadvantages, especially associated to insufficient compressive and/or bending strengths, poor abrasion resistance and esthetics (Kielbassa, Oehme, Shakavets, & Wolgin, 2021). The wear rate of conventional GICs was five times greater than amalgam and three times higher than composite-resin materials (Türkün & Kanik, 2016). Therefore, it was not surprising that their use was limited to only temporary treatment applications. However, nowadays, with various developments, GIC can show promising results even the stress-bearing sites (Fuhrmann, Murchison, Whipple, & Vandewalle, 2020).

Glass ionomers represent a group of materials with the same chemical properties. Classifications are made according to powder particle size, surface reactivity of powder particles, cure rate, and resistance to water loss and uptake (Mount, Makinson, & Peters, 1996). GIC can be classified as adhesive, base material, restorative, and root-canal filling paste according to their usage. According to their content; conventional glass ionomer cement (CGIC); hybrid glass ionomer cement (resin-modified glass ionomer cement (RMGIC)); polyacid-modified composite resins (compomers); high viscosity glass ionomer cement (HVGIC); giomers and nano-ionomers (Elmacı & Tunçdemir, 2020). The accepted classification is organized according to use in restorative dentistry (Mount, 1998) and can be schematized as follows (Table 1):

Table I. Classification of Glass Ionomer Cements

Glass Ionomer Cements (GIC)	
Type I	Luting and Bonding Cement
	1.5/1: Powder/Liquid ratio (P/L), low solubility and fast set
Type II	Restorative Cements
Type II.1	Restorative aesthetic. Auto-cure
	P/L: 3/1 or greater, low solubility, slow set, high wear resistance, translucent.
Type II.1	Restorative aesthetic. Resin-modified
	P/L: 3/1 or greater, light-initiated polymerization, acceptable wear resistance, translucent, higher strength
Type II.2	Restorative reinforced
	P/L: 3/1 or greater, higher physical properties, fast set, lower translucency
Type III	Lining Cement
	P/L: 1.5/1, low physical properties, can be resin modified, fast set
	Base Cement
	P/L: 3/1 or greater, higher physical properties, can be resin modified, fast set

Polymerization of Glass Ionomer Cements

After the GIC powder and liquid are mixed, it is turned into a stiff paste which progressively hardens by the acid-base reaction. When the acid attacks the glass particles, ions (Ca2+ and Al3+) are released. The combined effect of cross-linking of these ions on the polyalkenoic acid chains and the neutralization of the polyalkenoate molecules causes the cement to harden (J. W Nicholson, 2016). This happens in a short time, approximately 2-5 minutes after mixing.

This newly cured cement is not yet fully suitable for clinical service and its immature outer surface is highly sensitive to water changes. This surface may either be responsible for the advancement of an unpleasant chalky appearance with the formation of a network of microcracks by drying, or it may swell by absorbing water, causing a potential loss of network-forming ions and the consequent development of microcracks (Lohbauer, 2009). Covering the freshly poured cement with a layer of varnish or petroleum jelly inhibits this water movement and thus prevents the formation of a chalky appearance (Earl, Mount, & Hume, 1989).

Slower reactions persist over time. These are often stated as maturation involving various processes. Over time, an increase in ionic cross-linking and the proportion of bound water occurs. These variational curing steps lead to changes in glass ionomer cement properties, resulting in an increase in compressive and diametrical tensile strength over time, and translucency also improves (J. W Nicholson, 2016).

Clinical Applications

GIC has various indications in the clinic involving fissure sealing, Class V, and tunnel restorations (McLean, 1992). They are especially preferred in the treatment of non-carious cervical lesions (erosion: wear caused by chemical agents; abrasion: wear caused by physical agents and abfraction: wear caused by occlusal forces) (Francisconi, Scaffa, de Barros, Coutinho, & Francisconi, 2009). Since these lesions are characteristically close to the gingival margin, isolation is challenging and the presence of hypermineralized dentin is an obstacle to the application of adhesive systems. The use of GICs in non-carious cervical lesions is effective since the chemical bonding of GIC to the calcium of the tooth structure, and studies revealed that the retention of GIC in these lesions is up to 90-100% after 3 years of follow-up (Francisconi et al., 2009; Lambrechts et al., 1996).

Another usage of GICs is the sandwich technique in Class II cavities with deep margins. In the open sandwich technique, after the placement of GIC on the nearest part to the deep margin, an adhesive restoration is performed on it. Failure due to isolation is prevented and GIC can prevent the development of secondary caries by releasing fluoride. In an in vivo study, the durability of the open sandwich restorations was evaluated after 9 years and the failure rate was reported to be only %1.1 (Lindberg, van Dijken, & Lindberg, 2007).

The Atraumatic Restorative Treatment (ART) technique is a minimally invasive treatment method using GICs. This method is of great importance and provides convenience, especially in countries and regions where conditions such as the sufficient number of dental personnel, equipment, and devices cannot be met (Hilgert et al., 2014; Tekbas Atay & Koray, 2021). In addition, it is used as an alternative treatment method for children and individuals who are difficult to cooperate in the clinic. In this technique, after the excavation of carious dentin with hand instruments, a powder-liquid system conventional GIC is used. GICs are almost indispensable materials in the ART technique due to their biocompatibility, fluoride release and recharging properties, and chemical bonding to dental hard tissues (Bonifácio et al., 2013). Even though CIS is the only material that can self-bond to the dental tissue without any surface treatment, a pretreatment with weak polyalkenoic acid may increase the adhesive efficiency (Tekbas Atay, Ulu, & Oğuz Ahmet, 2013).

Resin-modified Glass-ionomer Cements (RMGIC)

To improve the mechanical properties of conventional GICs, resin-modified RMGIC containing hydrophilic monomers and polymers such as HEMA has been introduced. The chemical bond between the glass particles and the resin phase in its content allows them to have higher bending and tensile strength compared to conventional GICs (Yli-Urpo, Lassila, Närhi, & Vallittu, 2005). RMGICs show a photochemical curing mechanism called dual curing, which occurs with an acid-base reaction.

RMGICs have improved physical properties while retaining the clinical advantages of traditional GICs such as chemical adhesion to the dental hard tissues, fluoride release, and caries prevention. In addition, RMGICs have additional advantages such as ease of handling, control of working time, fast setting time, and less sensitivity to syneresis (Sidhu & Nicholson, 2016). However, their biocompatibility has been compromised as a result of the HEMA ingredients being released. It has been reported that even if it is polymerized as recommended by the manufacturer's instructions, residual monomer (HEMA) may be released and this may adversely affect the pulp biology at various levels (sensitivity, inflammation). It has been shown that RMCISs diffuse into the dentin and affect the pulp, especially within the first 24 hours after insertion, and as a result, they are more cytotoxic than GCISs (Park & Kang, 2020; Stanislawski, Daniau, Lauti, & Goldberg, 1999).

Polyacid-modified Composite Resin (Compomer)

Shortly after the introduction of RMGIC, compomers containing higher proportions of resin were produced to combine the advantages of composites and GICs. The polymerization of compomers is usually light-initiated which is similar to conventional composite resins. The initiator is camphorquinone with a blue light-sensitive amine accelerator at 470 nm (J. W. Nicholson, 2007). Compomers contain 70-80% composite resin and 20-30% GIC. Compomers do not contain water, hence the acid-base reaction and ion release occur only in a moist intraoral environment and allow the fluoride to be released from the material (Łagocka, Skoczyk-Jaworska, & Mazurek-Mochol, 2022). Although it contains 13% fluoride, fluoride release is quite low (Nezir & Özcan, 2022). Even though compomers contain GIC, a bonding agent is required for adhesion (Baygin, Korkmaz, & Arslan, 2012).

Having similar esthetic and physical properties to composite resin, the advantage of light-polymerization and ease of application is the reason for the preference of compomers, especially on cervical and anterior regions and deciduous teeth restoration (Demirci, Ersev, Topçubaşi, & Uçok, 2005; J. W. Nicholson, 2007). In the long term, constant water absorption

may lead to marginal discoloration. In a 3-year follow-up study of Class V cavities restored with compomers, it was observed that there was no change in other Ryge criteria except for color stability and marginal discoloration (Demirci et al., 2005).

High-Viscosity Glass-Ionomer Cements (HVGIC)

High viscosity glass ionomer cement (HVGIC) was developed by changing the powder/liquid ratio and powder particle of conventional GIC to advance inferior mechanical properties and make it applicable for load-bearing posterior region (Gok Baba, Kirzioglu, & Ceyhan, 2021). While the powder-liquid ratio is 3:1 or 4:1 in conventional GICs; in HVGICs, this ratio is 6:1 or 7:1 (Çelenk, Ataş, Ayna, & Günay, 2022). Therefore, HVGICs have higher wear resistance, surface hardness, and lower solubility. Encapsulated HVGICs standardize the powder/liquid ratio and ensure the optimum ratio and reduce the amount of porous caused by hand-mixing. According to the results of clinical studies, HVGICs showed similar success with amalgam restorations (Mickenautsch, 2016).

Manufacturers recommend applying a surface protector resin coating on HVGICs. This coating increases the gloss of the material and provides a smooth surface by filling the irregularities on the surface and reduces moisture sensitivity at the initial setting stage (Friedl, Hiller, & Friedl, 2011). İn vivo and in vitro studies revealed that the nanofiller-coated HVGICs showed a reduced wear rate than uncoated ones (Diem, Tyas, Ngo, Phuong, & Khanh, 2014). On the other hand, fracture toughness was not improved by coating (Fuhrmann et al., 2020). Besides, there are studies indicating that the resinous coating of HVGIC does not appear to be an efficient protection against long-term abrasion since the coated parts are also abraded (Kielbassa et al., 2021).

In a 2-year follow-up evaluation, HVGIS represented a similar success rate to conventional resin composites in Class II cavities of permanent teeth (Menezes-Silva, Velasco, E, Bastos, & Navarro, 2021). In an in vivo study comparing traditional GICs and HVGICs, both materials had similar success rates in class 1 cavities. However, the clinical performance of HVGIC was superior in Class 2 cavities. In addition, a decrease in clinical performance was observed as the number of restoration surfaces increased (Klinke et al., 2016). Hilgert et al. stated that the survival rates of amalgam and HVGIC restorations in primary molars over the total period of 3 years were quite similar. Moreover, single-surface restorations yielded greater survival rates than multi-surface restorations in accordance with the previous studies (Hilgert et al., 2014).

Zirconia Reinforced Glass-Ionomer (Zirconomer)

Zirconomer which is also called white amalgam is developed to display toughness almost consistent with amalgam (Salman et al., 2019). The addition of nano-sized zirconia fillers to glass component strengthens the structural integrity of the restoration, improves its compressive and flexural strengths, thereby allows its use in load-bearing sites and enhance aesthetic properties (Albeshti & Shahid, 2018; Patel et al., 2015).

In a study conducted by Kukreja et al. fluoride release of Zirconomer and GIC was compared at 6 hours to 14 days and higher fluoride levels were observed in the Zirconomer group at all time intervals (Kukreja et al., 2022). In a study comparing the surface roughness of GIC and Zirconomer after the application of acidic beverages, the surface of the Zirconomer was less affected compared to GIC and that indicates the acid-resistant surface of Zirconomer (Nugraeni, Hartami, & Nurul Ummah, 2020).

Giomer

In recent times, the increasing interest in minimally invasive dentistry has accelerated the advancement of materials with adhesive properties, fluoride (F) release, and higher mechanical and aesthetic properties. For that purpose, by combining the characteristics of the GIC and composite resins, hybrid products knowns as giomers (Glass ionomer + Polymer) have been developed (Rusnac et al., 2019). Giomers can be defined as a special class of composites that can provide protection against caries and provide functional and aesthetic results thanks to the presence of pre-reacted glass filler particles in the matrix of the composite material (Kimyai, Savadi Oskoee, Ajami, Sadr, & Asdagh, 2011). Giomers (unlike GICs and RMGIs) require a resin bonding system after acid etching like compomers.

In a cytotoxicity study conducted on in vitro fibroblast cells, giomers represented better biocompatibility than conventional GIC and resin composite (Tamilselvam, Divyanand, & Neelakantan, 2013). It is known that the polishability of restorations made with giomer in cervical cavities is higher than RMGICs. The superior polishability of giomers is not surprising since their content is similar to composites (Jyothi, Annapurna, Kumar, Venugopal, & Jayashankara, 2011).

In an in vitro study comparing the flexural strengths of giomers and RMCIS, the flexural strength of the giomer was found to be significantly higher (Sulaiman, Yeo, & Chong, 2007). On the other hand, when the microleakage of different restorative materials was compared, the material showing the most microleakage was giomer, followed by RMCIS, colored compomer (Yadav, Rehani, & Rana, 2012).

Nano-ionomer

The manufacturing of structures and materials in mamoscale (the range of 0.1 nm to 100 nm) (by various methods is called nanotechnology and can also be used for the development of restorative materials (Korkmaz, Ozel, Attar, & Ozge Bicer, 2010). Recently, a light-cured RMGIC containing nanoparticles has been introduced and is called a nano-ionomer. The nano-ionomer consists of 69% by weight nano-sized fillers such as silane-treated silica and zirconia together with fluoroaluminosilicate glass, thereby having enhanced mechanical properties such as wear resistance and improved surface polishability (Bollu et al., 2016). In a study by Moghimi et al., the wear rate of nano-ionomer was less than conventional GIC and RMGIC (Moghimi, Jafarpour, Ferooz, & Bagheri, 2022).

Nano-Ionomer can bond chemically to the tooth structure however there are studies in the literature that show a tight interface between the nano-ionomer and dentin (without any signs of dentin demineralization or hybrid-layer formation) with the primer application prior to restoration (Coutinho et al., 2009). Moreover, Bollu et al. stated that the etching with phosphoric acid can also increase the shear bond strength between nano-ionomer restorative and enamel (Bollu et al., 2016).

In a study comparing the marginal adaptation of several restorative materials in Class V, the results indicated that nano-ionomer and RMGIC have better marginal adaptation than the giomer and the nano-ionomer showed the least microleakage (Bollu et al., 2016). Similarly, Salman et al. reported that nano-ionomer and RMGIC have lesser microleakage scores than Zirconomer and the highest score was belong to the giomer (Salman et al., 2019). In a study investigating the fluoride release amount, the fluoride release from the nano ionomer was reported to be higher than RMGIC and compomer (Neelakantan, John, Anand, Sureshbabu, & Subbarao, 2011).

Glass ionomer cement containing bioactive glass

Bioactive glasses (BAG) are structures consisting of calcium, sodium, phosphorus, and silicon oxides to which bone minerals can be chemically bonded. They can be used in dentistry for bone regeneration, dentin remineralization, and the improvement of restorative materials (Yli-Urpo, Lassila, et al., 2005). The addition of BAG to GIC and RMGIC showed promising results in terms of dentin demineralization (Mousavinasab, Khoroushi, Keshani, & Hashemi, 2011). However, studies revealed that the addition of BAG particles to GIC decreases compressive strength and the modulus of elasticity probably due to the loose attachment of BAG particles to the GIC matrix (Park & Kang, 2020). On the other hand, previous studies evaluating surface characteristics and mechanical properties of

RMGI-BAG reported a uniform and homogeneous layer of deposits on the surfaces whereas RMGI exhibited fewer deposits but there were cracks on the surface (Yli-Urpo, Lassila, et al., 2005). BAG also has an antibacterial effect as its high pH of aqueous solutions.

It is known that the amount of BAG added to the GIC also affects the mechanical properties. The addition of BAG to GIC and RMGIC can increase mineral deposition in dentin, but the rate of BAG added should be considered, as an increase in BAG content leads to a decrease in mechanical properties (Yli-Urpo, Närhi, & Närhi, 2005).

Glass ionomer cement containing hydroxyapatite

Hydroxyapatite [HAp: $Ca_{10}(PO_4)_6OH_2$] has a structure similar to human tooth and bone structure and has superior biocompatibility. Studies have announced that the addition of HAp to GIC improves mechanical properties such as toughness, hardness, flexural strength, and modulus (Arita et al., 2011; Bali, Prabhakar, & Basappa, 2015). The strong ionic bond formed between the calcium ion of the tooth structure and the apatite crystal of the cement is responsible for the strong adhesion of the nano-HAp-containing GIC to the tooth surface (Lucas, Arita, & Nishino, 2003). Besides, lessening the HAp particle size from micro to nano scale significantly increases the surface area and enhances infiltration into the dentin and enamel pores where the crystals are demineralized; this can improve the bonding at the tooth-ionomer interface (Lee et al., 2010).

Glass Carbomer

Glass carbomer is a new material that contains nanosized reactive glass treated with dialkyl siloxanes (European Patent 20040748628) and nanocrystals of calcium fluorapatite as a secondary filler (Cehreli, Ebru, Yalcinkaya, & Cehreli, 2013). These nanocrystals act as nuclei for the remineralization process, initiate the formation of fluorapatite and facilitate the strengthening of the material (Subramaniam, Girish Babu, & Jayasurya, 2015).

Glass carbomers can be placed in the cavity with the bulk technique without any surface preparation. Although glass carbomer can be hardened by auto polymerization, manufacturers recommend photopolymerization with a high-energy dental polymerization light which allows for shorter polymerization time and improved clinical outcomes. Besides, coating the surface with a varnish is also recommended. The surface varnish provides a protective layer on the cement with its monomer-free content and prevents the cement from saliva contamination in the early stage, allowing the polymerization to take place properly, and preventing the dehydration

of the filler in the advanced stage (Buldur & Sirin Karaarslan, 2019). In accordance with this, Cehreli et al. stated that the uncoated glass carbomer represented higher microleakage scores. Furthermore, coated glass carbomer yielded similar microleakage scores compomer (Cehreli et al., 2013).

A study investigating the microleakage of conventional GIC, HVGIC, zirkonomer, and glass carbomer observed no significant differences between materials in enamel margins. However, HVGIC and Zirkonomer showed less microleakage than glass carbomer in dentin margins. Also, the same study revealed no significant difference between the shear bond strength of this material (Meral & Baseren, 2019). In a 3-year follow-up study, the clinical success of compomer and GIC was found to be higher than glass carbomer for Class I and Class II cavities in primary molars (I. C. Olegário, Hesse, Mendes, Bonifácio, & Raggio, 2019).

Although the previous studies claim that having nanosized powder particles and fluorapatite improves the compressive strength and resistance of glass carbomer, a study conducted by Olegário et al. showed that the HVGIC had higher bond strength and Knoop hardness values than the glass carbomer in ART restorations (Isabel Cristina Olegário et al., 2015). On the other hand, Gorseta et al. have reported that thermo-light polymerization will contribute to the superior mechanical properties of glass carbomer (Gorseta, Borzabadi-Farahani, Moshaverinia, Glavina, & Lynch, 2017). A study investigating the nano hardness and surface roughness of HVGIC, compomer, and glass carbomer revealed that the glass carbomer has similar properties to the compomer in terms of nanomechanical structure (Altan et al., 2016).

A study conducted by Lopes et al. reported that the surface roughness and fluoride release of glass carbomer were similar to GICs (Lopes, Galvan, Chibinski, & Wambier, 2018). Nevertheless, Kaynar and Dönmez compared the clinical performance of glass carbomer and composite resin for 12 months with modified USPHS criteria and observed a significant difference between the two materials in terms of marginal discoloration and adaptation. A study comparing the color stability and surface roughness of glass carbomer and a micro-hybrid resin composite showed that the glass carbomer did not provide surface properties like the micro-hybrid resin composite (Bal, Karaarslan, Buldur, Agaccioglu, & Demir, 2022). The results of this study indicated that the physical properties of glass carbomer still need to be improved (Kaynar & Dönmez, 2022).

Conclusion

The clinic usage of GICs is recommended due to chemical adhesion, fluoride release, and moisture tolerance. Disadvantages such as low wear resistance, inferior mechanical properties, and esthetics that limit their clinical use can be overcome by various improvements. Consequently, it was concluded that the limited use of glass ionomer cements as a permanent restorative material alternative to amalgam and composite resin will increase in light of current developments.

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CHAPTER 5

THE BIOLOGY AND FUNCTIONS OF EXOSOMES: DIAGNOSTIC AND THERAPEUTIC APPLICATIONS IN NORMAL AND PATHOLOGICAL CONDITIONS

İlknur ÇINAR AYAN[1]

1 Necmettin Erbakan University, Meram Faculty of Medicine, Department of Medical Biology, KONYA, TURKEY ORCID: https://orcid.org/0000-0002-8763-0480 E-Mail: ilknrcnar@gmail.com

Introduction

Extracellular vesicles (EVs), which until recently were thought to be responsible only for removing cell wastes from the cell, are lipid-bound vesicles secreted out of the cell (Zaborowski et al., 2015). EVs are nano-sized structures released from cells to the extracellular space, which can transfer cargo containing many components of the cell, such as nucleic acids (DNA and RNA), lipids, proteins, and various metabolites to enable intercellular communication (Yáñez-Mó et al., 2015; Doyle and Wang, 2019). EVs deliver their cargo to target cells and tissues through many body fluids (such as saliva, urine, blood, lymphatic system, breast milk, cerebrospinal fluid) (Boukouris and Mathivanan, 2015). EVs play important roles in many physiological and pathological processes using different signaling pathways (Rezaie et al., 2022). It is known that EVs consist of 3 main subtypes according to their intracellular origin, biogenesis mechanisms, secretory pathways from the cell, size, cargo contents and functions (Borges et al., 2013; Doyle and Wang, 2019).

1. Microvesicles (MVs)
2. Apoptotic bodies
3. Exosomes

1. Microvesicles (MVs):

MVs are vesicular in the 100-1000 nm size range formed by budding or pinching directly out of the cell membrane (Teng and Fussenegger, 2020). MVs are also known as ectosomes or microparticle-derived vesicles. Exosomes and microvesicles are secreted from healthy cells into the extracellular space (Zhang and Yu, 2019). Membrane proteins such as CD42 (integrin GPB) and P-selectin are localized abundantly on microvesicles (Heijnen et al., 1999). Microvesicles can be detected by flow cytometry and electron microscopy methods, and other proteins identified in MVs are cytoskeletal proteins, HSPs (heat shock proteins) (Doyle and Wang, 2019). It has been understood that MVs are not only a means of removing unwanted residues within the cell, but also one of the mechanisms responsible for cell-cell communication between both nearby and more distant cells (Rak, 2010).

2. Apoptotic bodies:

Apoptotic bodies are released from the plasma membrane in the late stage of apoptosis, a programmed cell death (Zhang et al., 2019). Apoptotic bodies are heterogeneous populations of Membrane vesicles, ranging in size from 50 to 5000 nm (Théry et al., 2001; Borges et al., 2013). Apop-

totic bodies occur when the plasma membrane detaches from the cytoskeleton following cell contraction (Wickman et al., 2012). Compared to the content of exosomes and MVs, apoptotic bodies contain fragmented DNA and organelles differently. Accordingly, the organelle-specific proteins contained in apoptotic bodies are also found at higher levels (Théry et al., 2001; Borges et al., 2013; Doyle and Wang, 2019). While apoptotic bodies are detected by flow cytometry and electron microscopy methods, their isolation is also performed by ultracentrifugation (10000g-20000g) method (Zhang et al., 2019).

3. Exosomes

Discovery of exosomes

Exosomes were originally thought to form by budding or pinching directly from the plasma membrane, like microvesicles. However, with subsequent studies, it was discovered that small-sized vesicles were formed by budding inward in an endosome in the cell and then fused with the plasma membrane, causing the formation of a multivesicular body (MVB) (Kowal et al., 2014). These small vesicles have been termed exosomes (Johnstone et al., 1987). When exosomes were first discovered, they were thought to be cellular waste formed due to cell damage and by-products of the normal cellular homeostasis process (Simpson et al., 2009). Today, it has been understood that exosomes surrounded by a double-layered phospholipid membrane are functional vehicles that carry cargo such as proteins, lipids, different types of RNA and DNA (Waldenström et al., 2012; Valadi et al., 2007). Depending on the cargo they carry, exosomes play a vital role in many cellular processes related to intercellular communication, such as signal transduction and immune response (Greening et al., 2015; Gangoda et al., 2015). Endosomally formed exosomes are abundant in many body fluids (such as urine, semen, bronchial fluid, breast milk, lymph fluid) since they can be secreted by all cell types (Doyle and Wang, 2019).

Exosome biogenesis

Exosome formation and MVB regulation are carried out by ESCRT (endosomal sorting complexes required for transport) pathway proteins and ESCRT-related auxiliary proteins (ALIX, TSG101, HSC70 and HSP90β) (Géminard et al., 2004). Therefore, these proteins must be common in all exosomes and in all cells. Since these proteins are found in all exosomes, they are defined as “exosomal marker proteins” (Trajkovic et al., 2008). In the process of exosome biogenesis, the early endosome occurs firstly, induced by ceramide on the cytosolic surface of the plasma membrane via endocytosis. Many intraluminal vesicles (ILVs) occur during the con-

version of early endosomes to late endosomes with the contibution of the endoplasmic reticulum and golgi complex (He et al., 2018). ILV which contains many cytosolic components, comes together and the endosome membrane is closed and endosomes are transformed into multivesicular bodies (MVBs). As a result, MVBs either associate with lysosomes to be degraded or fuse with the plasma membrane to release their ILVs into the extracellular space as exosomes (Bozkurt, 2018; He et al., 2018; Doyle and Wang, 2019; Kalluri and LeBleu, 2020).

Cargo transfer to ILVs is accomplished through two mechanisms, ESCRT-dependent and ESCRT-independent (Stuffers et al., 2009; de Gassart et al., 2004). Recognition of ubiquitinated proteins and transport into the vesicle is carried out through an ESCRT-dependent mechanism. ESCRT consists of 4 complexes (ESCRT-0-1-2-3) and ESCRT-related accessory proteins (Farooqi et al., 2018). The ESCRT-0 complex is responsible for the collection of ubiquitinated proteins and clathrin cargoes to the endosomal membrane. ESCRT-1 and 2 trigger the budding process and help dissociate cargo proteins prior to formation of ILVs in MVBs. ALIX and TSG101 ESCRT- accessory proteins are responsible for the stability of the structure. ESCRT-3, on the other hand, causes the membrane to fold inward, allowing the budding vesicle to separate from the membrane (Henne et al., 2011; Ha et al., 2016; McGough and Vincent, 2016; Farooqi et al., 2018). It is thought that the independent mechanism from ESCRT is dependent on the sphingomyelinase enzyme formed from ceramide and tetraspanin instead of ESCRT (Stuffers et al., 2009). These ceramide-rich lipid rafts of the cell membrane and tetraspanin cause ILV formation (Colombo et al., 2013).

Ceramide plays an important role in ESCRT-independent exosome biogenesis by promoting budding due to its structure (van den Boorn et al., 2013). When MVBs fuse with lysosomes, either their ILV contents are degraded or they can fuse with the plasma membrane, which will allow them to be released into the extracellular environment as exosomes. It has been understood that Rab GTPases function in the transport of MVBs to the plasma membrane and the release of exosomes into the extracellular space (Kowal et al., 2014; Farooqi et al., 2018). However, it has been demonstrated through RNA interference-mediated knockdown studies that also many protein families such as SNARE and clathrin play a role in the fusion mechanism with the plasma membrane, which triggers exosome release (Cai et al., 2007; Ostrowski et al., 2010).

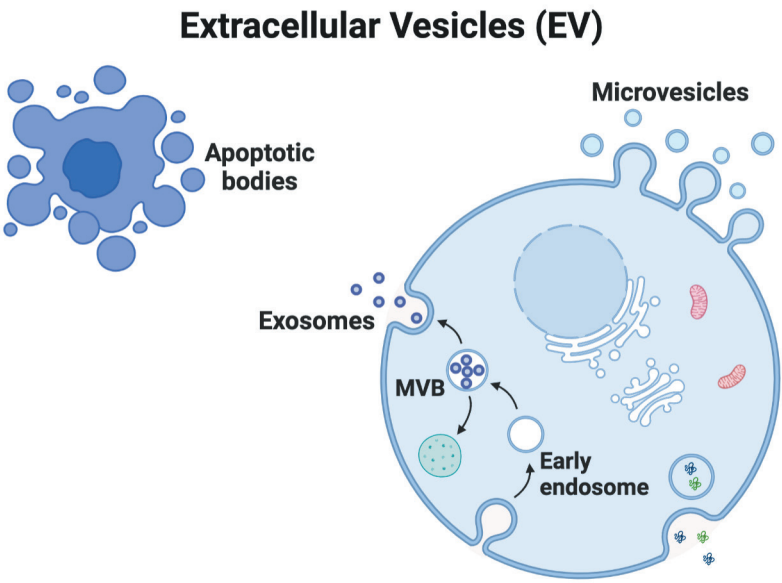

Figure 1: *Three subclasses of Extracellular Vesicles and biogenesis of Exosomes (Created with BioRender.com).*

Physiological and pathological functions of exosomes

Exosomes were initially thought to be excretory vehicles responsible for the removal of intracellular debris, senescent and excess organelles (Doyle and Wang, 2019). Recent studies have shown that exosomes can be found everywhere in the organism and can be secreted by all mammalian cells. The widespread pattern of exosomes in the organism also shows that it plays a role in intercellular communication by transmitting information in a cell to all cells and tissues, regardless of whether it is close or far (He et al., 2018). The cell that receives the exosome by internalization regulates the related gene expression and functions and, creates a response to the transmitted message. Exosomes are transported to target cells, tissues and organs in body fluids (such as amniotic fluid, sperm, breast milk, saliva). Therefore, exosomes function in the transmission of both physiological and pathological signals (Qin and Xu, 2014; Simpson et al., 2008; Santonocito et al., 2014). It has also been determined that, unlike the paracrine and endocrine effects, the exosomes secreted from pancreatic cancer cell lines also exert an autocrine effect on themselves (Ristorcelli et al., 2009).

The function of exosomes released from cells differs depending on the origin of the cell. For example, in the nervous system, exosomes play a role in tissue regeneration and communication between nerve cells by

helping myelin formation and neuronal survival under physiological conditions (Fauré et al., 2006; Lachenal et al., 2011). Exosomes play a role in the pathogenesis of many neuroinflammatory processes as well as the physiological conditions of brain functions. Exosomes carry out the induction of the inflammatory system through many pathways; It provides induction of macrophages to increase pro-inflammatory cytokines and TNF secretion (Bhatnagar and Schorey 2007; Vega et al., 2008). Exosomes mediate adaptive and innate immune responses involved in antigen presentation and delivery of MHC I and MHC-II molecules (Sun et al., 2013). It also plays a role in the maturation of dendritic cells (DC) (Skokos et al., 2003). It is known that exosomes have important effects on many stages of reproduction and development, such as the formation of healthy germ cells, fertilization and the implantation process after embryo formation (Yáñez-Mó et al., 2015). Exosomes also function in maintaining homeostasis by promoting cell proliferation. Exosomes originating from hepatocytes can perform liver regeneration by generating sphingosine-1-phosphate with sphingosine kinase 2 transfer in target cells (Nojima et al., 2016). It was determined that tumor cells secreted 10 times more exosomes compared to normal cells (Shao et al., 2016). The metastasis process was triggered by the transfer of tumor-derived exosomes to benign tumor cells. In addition, tumor-derived exosomes play an important role in many stages such as invasion, migration, angiogenesis, drug resistance related to the phenotype and character of the tumor (Zomer et al., 2015; Shao et al., 2016).

Role of exosomes in diagnosis and treatment

The “fingerprint” features of exosomes allow for the differentiation, characterization and isolation of exosomes from other vesicles (such as microvesicules, apoptotic bodies). However, the biggest obstacle to the widespread clinical use of exosomes is the inability to fully standardize the isolation methods (Zhang et al., 2018). Differential centrifugation, ultracentrifugation and ultra-filtration methods are commonly used for exosome isolation. The most important reasons underlying the failures in isolation methods are the complex body fluids from which exosomes are obtained, their similarities with other EVs, and the heterogeneity of exosome structures among themselves (Smith et al., 2015). It is known that exosomes are abundant in all body fluids. The specific cell content of exosomes (lipid profile, protein, RNA and DNA) and the “fingerprint” features of membrane proteins can represent the physiological state of the cell from which they originate. Therefore, exosomes obtained from body fluids can be used as important biomarkers for the diagnosis of various physiological conditions and pathological diseases. For example, exosomes expressing

CD63 and Caveolin-1 in plasma can be used as a biomarker for the diagnosis of melanoma (Logozzi et al., 2009).

The mRNA transcript that carries out ZFY (zinc finger protein) protein expression, which is expressed in exosomes originating from the amniotic fluid, can be used to determine the sex of the fetus. An advantage of the presence of exosomes in many body fluids is that the patient can be diagnosed with non-invasive "liquid biopsy" type methods and even allows direct monitoring of the patient's response to treatment (Sonoda et al., 2009). In addition, exosomes may contain lncRNAs of approximately 200 nt in length that cannot be converted to protein. These RNAs are used as biomarkers in the diagnosis and processing of various diseases. For example, expression levels of exosomal lncRNA and miR-217 in CRC (colorectal cancer) patient serum vary according to the prognosis of the disease (clinical stage/lymph node/distant metastasis) (Yu et al., 2017).

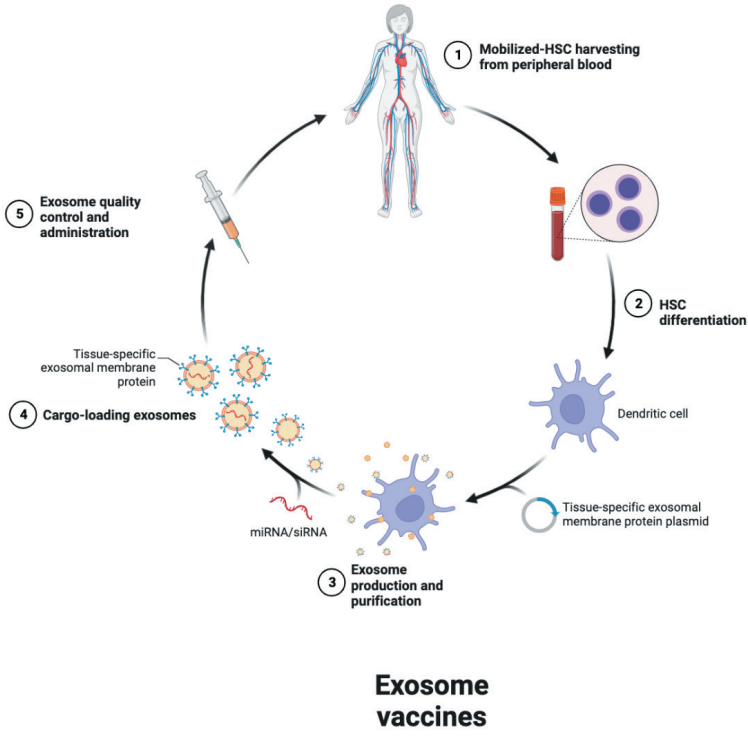

Figure 2: *Diagram of cancer vaccines, one of the clinical applications of Exosomes (HSC: hematopoietic stem cells)(Created with BioRender.com).*

Recently, studies on new nanotechnology delivery systems have been focused on in order to increase the specificity of drugs to target cells and

tissues, their duration of action and to minimize their toxicity on normal cells (Kotmakçı and Çetintaş, 2015). For example, the chemotherapy agent doxorubicin is encapsulated in liposomes and paclitaxel is encapsulated in protein-based nanoparticles (Liu et al., 2016). However, after various applications, it has been understood that drug delivery systems exhibit disadvantages such as low circulating time and increasing toxicity independent of increasing drug dose due to dose increase. The biggest advantages of exosomes are that they show high biocompatibility to the target because they originate from cell membranes, have much lower toxicity, and can easily penetrate tissues and organs without being caught by the immune system (Bang and Thum, 2012). For this reason, exosomes are considered as one of the most suitable tools in the treatment of many diseases. When many preclinical and clinical trials and studies are evaluated together, it is seen that the most effective exosome applications are the use of biomarker, exosome therapy, exosome drug delivery system and exosome vaccines (cancer vaccines) (Kalluri, 2016) (Figure 2).

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CHAPTER 6

PELVIC CONGESTION SYNDROME

Hüseyin DEMİRTAŞ[1], Ali DOĞAN[2]

1 Inst.Dr., Gazi University Medical Faculty Hospital Cardiovascular Surgery Department ORCID IP: 0000000257101385
2 Ra.Dr., Gazi University Medical Faculty Hospital Cardiovascular Surgery Department ORCID IP: 0009000602553989

INTRODUCTION

Many women around the world suffer from Pelvic Congestion Syndrome (PCS), which frequently goes undiagnosed. A comprehensive approach is required for an accurate diagnosis of gynecological symptoms with a vascular background. Atypical varicose veins and chronic pelvic pain have this as a relevant cause. Imaging studies that have been correlated with the clinical presentation form the basis of the diagnosis. Possible causes of PCS include genetic proneness, anomalous anatomy, hypertension, hormonal factors, vein wall damage, valve dysfunction, reversal of the blood flow, and dilatation, even though its exact cause is still unknown.

Chronic pelvic pain (CPP) is described as pelvic or abdominal discomfort/ache that lasts for more than six months also is either intermittent or persistent(1). CPP affects many women and has a significant financial impact on health budgets (2). It is severe enough to require treatment or leave one with a functional disability.

PCS is frequently an underdiagnosed cause of chronic pelvic pain in female patients. It can happen on its own or in conjunction with lower extremity venous insufficiency, vulvar varicosities, or both. The systematic review written by Ahangari states that its prevalence varies between 6% and 27% globally (3). According to some authors, PCS may contribute to CPP in as many as 30% of women (4,5).

39% of women or more say they eventually experience pelvic pain (6). Roughly 20% of all laparoscopic surgeries are carried out for chronic pelvic pain, and 2% to 10% of all gynecological clinic consults are for pelvic pain. Around 7 million of the estimated 10 million women who have this illness choose not to receive treatment.

The effects of CPP on the economy are astounding. The estimated annual medical expense for diagnosing and treating CPP is $1.2 billion. These patients are estimated to cause a loss of $15 billion in productivity each year. For as many as 61% of patients, there is no known cause of their discomfort (7). According to estimates, the prevalence of persistent pelvic discomfort varies from 5.7% in Austria to 26.6% in Egypt (8). 3.8% of women in the UK experience it on an annual basis, making it a common presentation in primary care. This proportion is likened to back ache (4.1%), and asthma (3.7%) (9).

HISTORY

Richet noted a connection between varicose veins in the utero-ovarian plexus and persistent pelvic pain in the middle of the 19th century. He also noted the occurrence of pelvic varices (10). Taylor first identified pelvic

venous enlargement as a contributing factor to chronic pelvic discomfort in 1949 (11). This was later confirmed by Hobbs (12) and Lechter in 1976 and 1985, respectively (13). PCS was first outlined by Beard et al. in 1984. In premenopausal, multiparous women who have a history of persistent pelvic pain lasting longer than six months, it is characterized by obvious pelvic venous congestion on venography (14). Having persistent symptoms such as perineal heaviness, pelvic pain, postcoital pain, and the need to urinate immediately that are brought on by ovarian and/or pelvic vein reflux and/or obstruction and that may be accompanied by perineal, vulvar, and/or lower extremity varices was described as having pelvic congestion syndrome in the VEIN-TERM transatlantic interdisciplinary consensus document from 2009 (15).

RISK FACTORS

Premenopausal and typically multiparous patients have PCS. The risk factors for PCS are; being multiparous, having a positive family history of pelvic pain, abnormalities in pelvic venous anatomy, hormonal pathologies, estrogen therapy, varicose veins in the lower limbs, having had pelvic surgery in the past, prolapsed uterus, heavy lifting, or prolonged standing (16-18).

According to Nanavati et al., women with PCS have a higher likelihood of having a normal BMI than being obese (19).

Although a positive family history of pelvic ache is a risk factor, it is still unclear whether there is a genetic predisposition. Mutations in the FOXC2, NOTCH3, TIE2, and thrombomodulin genes might be involved in the pathogenesis (16,20).

ANATOMY-PHYSIOPATHOLOGY

A web of interconnected veins drains the bladder, uterus, vagina, and rectum. Within the broad ligament, the uterine fundus drains to either the ovarian or the uterine plexus (utero-ovarian and salpingo-ovarian veins). The internal iliac veins are branches of the uterine veins, which receive drainage from the vagina and lower uterus. Just below the right renal vein, the right ovarian plexus drains into the right ovarian vein, while the left ovarian plexus empties into the left ovarian vein, which empties into the left renal vein. Seldom if ever, the right ovarian vein drains into the right renal vein. Vulvoperineal veins empty into the circumflex femoral vein, the saphenous vein, the internal pudendal vein, the inferior gluteal vein, the external pudendal vein, and finally the femoral vein. The valves typically exist within the major ovarian vein trunks, but they are much less frequently (only about 10% of cases) found within the IIVs. 15% of women

have ovarian veins without valves, with the left side being more common. When valves are present, they are typically found in the vein's distal section, close to the point where the renal veins converge. However, in 40% and 35% of cases, respectively, the left and right ovarian vein valves are ineffective when present (20).

Venous obstruction can lead to secondary venous congestion. May-Thurner Syndrome (MTS), a state in which the left common iliac vein (CIV) is compressed because it is situated between the right common iliac artery and the lumbar spine, may play a role in the development of secondary PCS (21).

The degree of stenosis, according to Costa et alstudy .'s using magnetic resonance imaging published in 2020, is unrelated to symptoms, and a sizable portion of both symptomatic and asymptomatic patient populations exhibit compression of the left CIV (22).

The Nutcracker syndrome (NCS), another anatomical variation that may cause secondary PCS, is characterized by compression of the LRV between the aorta and a vertebral body in the posterior type and between the aorta and the superior mesenteric artery in the anterior type (23). Some medical professionals believe that the patient's position lying on her back during the imaging may have led to an overdiagnosis of the cause. This was also observed in a study where the degree of stenosis in intravascular ultrasound was significantly influenced by posture (In the supine position, 55% of the LRV had significant stenosis, compared to 10% when lying on the left side.) (24).

The primary potential problem in PCS appears to be reflux caused by ineffective valves in the pelvic and ovarian veins (25). Uncertain mechanisms underlie the valves' deterioration. On the one hand, there might be fundamental modifications to the structure of the valves, which could result in dysfunctional valves, therefore developing reflux, and finally venous enlargement. On the other hand, there might be underlying structural issues with the vein wall that cause dilated veins and, as a result, distorted valves that cause reflux. Regardless of the initial triggers, persistent venous dilatation leads to inflammation, which further erodes the structure of the valve and significantly increases reflux. The pelvic varicosities linked to PCS can also be attributed to a disruption of vein wall integrity. The expression of matrix metalloproteinases is increased by venous hypertension, and these enzymes deteriorate the underlying endothelium and smooth muscle (26). Because these modifications make it more difficult for veins to constrict and relax, venous pressure rises and endothelial cell damage is promoted by triggering leukocyte infiltration and inflammation. Chronic venous distention and reflux result from this. PCS is frequently associated

with estrogen hyperstimulation, though its importance is unclear. Ultrasound can detect polycystic ovaries in up to 50% of PCS patients who do not have hirsutism or amenorrhea (27).

The fact that PCS symptoms worsen during menstruation, that PCS is more common in multiparous and premenopausal women, that hormonal replacement therapy has beneficial therapeutic effects on PCS symptoms (28), and that sex hormones are highly concentrated in the blood that refluxes to the groin (29) all point to the pathophysiology of symptomatic varicosities being significantly influenced by hormonal factors. Progesterone weakens venous valves, while by causing venous dilatation through the release of nitric oxide, estrogen is known to weaken veins. These effects may help to encourage the development of dysfunctional ovarian and pelvic veins and the ensuing reflux. PCS is by definition accompanied by pelvic varicosities linked to CPP. The previous discussion did highlight some potential factors that could influence the development of varicose veins, but it was insufficient in explaining the causes of pelvic pain. Even though venous distention does not always result in pain, the stretched and stagnant ovarian and pelvic veins might stimulate pain receptors within the veins (30) resulting in diffuse pain brought on by the low nociceptive afferent density in the viscera. The fact that neuropathic painkillers like amitriptyline and gabapentin work better than opioid or non-steroidal analgesics at reducing pelvic pain provides evidence that ovarian vein distention causes pain receptor activation (31). Furthermore, compared to patients with isolated lower limb varicose veins, patients with pelvic and lower limb varicose veins experience more pain (32). According to these studies, there is a direct correlation between the degree of dilatation of the vein and reflux and the intensity of pain. Another theory for pain in PCS is the discharge of neurotransmitters from dilated pelvic veins' walls (33-35). In symptomatic PCS patients, elevated levels of substance P have been found (35). Additional research demonstrates that substance P antagonists reduce pelvic pain, further demonstrating that substance P is a factor in the symptoms of PCS (36). The neurotransmitter calcitonin gene-related peptide (CGRP), which is connected to the reproductive tract's sensory nerves and autonomic feedback, has also been linked to pain. Studies show that PCS is associated with CGRP supersensitivity because CGRP infusion worsens pelvic aches in women with PCS. The fact that medroxyprogesterone acetate therapy reduces ache by preventing neurotransmitter discharge provides additional proof that neurotransmitter discharged from dilated veins causes pelvic ache (34-37).

Pelvic pain is brought on by external mechanical compression, which also triggers the release of nociceptive factors and the activation of pain receptors. Within the cavity, the anatomy of the pelvic structures is compact. By compressing nearby nerves against surrounding anatomical struc-

tures, ovarian and pelvic vein dilatation and the localized inflammation they cause can cause ischemia and the visceral pain of PCS (37).

SYMPTOMS

In earlier PCS theories, the pain was characterized as a characteristic feature of PCS and was described as chronic, dull, and either unilateral or bilateral (38,39). Today's PeVD concept, put forth by Meissner et al., recognizes the problem, as similar levels of chronic venous insufficiency can result in a variety of symptoms, and even symptoms that are the same across patients can have different underlying pathophysiologies (40). The only sign of PVI may be superficial varicose veins, which can happen even when there is no pelvic pain (40, 41).

Prolonged periods of standing, or being seated as well as mechanisms that raise abdominal pressure, such as lifting heavy and pregnancy, are described in the literature as aggravating factors for pelvic pain (41-44). Additionally, the pain gets worse during and after sex. According to Osman et alresearch, 's dyspareunia caused by endometriosis is frequently accompanied by deep penetration, whereas PCS pain is frequently made worse by sexual activity and results in throbbing pain afterward (45). To help patients suspected of having PCS differentiate between different types of dyspareunia, research, and data are desperately needed. Pain typically gets worse throughout the day, as well as the days leading up to and during the menstrual cycle. Typically, lying down will lessen your symptoms (46). Unusual aches can also be sudden or may be localized elsewhere like the lower back, legs, hips, or abdomen. The pelvic veins form a web around the organs and have numerous connections between them, so these unusual patterns can be seen. Patients with PVI might present with atypical varicose veins of the proximal regions of the thighs as well as gluteal, vulvovaginal, and suprapubic perineal varices (47). Vulvar varices can occur in as many as 24–40% of PCS patients (48,49). The varicose veins that are treated surgically frequently come back when the abnormal pelvic venous flow is not treated (50). Up to 80% of patients with pelvic venous insufficiency may have associated lower limb venous insufficiency (50,51).

With age, leg symptoms like pain, edema, and heaviness are reported more frequently. Infertility and venous leg ulcers may also be caused by PCS (52,53). Urinary symptoms like urgency or dysuria may develop during PCS as a result of perivesical varicosities in the bladder. Additionally, PCS can resemble hip osteoarthritis or a mons pubis abscess (54,55). Headache, lumbosacral neuropathy, dysmenorrhea, swollen vulva, leg heaviness, vaginal discharge, persistent genital arousal, rectal discomfort, and nonspecific symptoms like distension of belly, vomiting and nausea are some additional PCS manifestations (55).

DIAGNOSIS

A distinctive pattern of varicosities, whenever they are accompanied by specific pelvic symptoms, can help pinpoint patients who need additional diagnostic testing. While imaging studies cannot diagnose PCS, they can confirm the presence of this clinical pattern. For pelvic varicosities, various imaging modalities adhere to various diagnostic standards. When a patient has suspected PCS, pelvic doppler ultrasound is the first imaging modality used. Although transabdominal and transvaginal ultrasound can both be used, the transvaginal approach with Doppler evaluation is superior because it allows for dynamic analysis of blood flow through complex pelvic veins that improves pelvic venous plexus visualization. Standing or performing a Valsalva maneuver can be used to image patients using ultrasound, which highlights venous filling and improves the ability to see pelvic varicosities. A straight tubular structure with a typical diameter of 4 mm is what the normal venous plexus looks like. In patients with pelvic varicose veins, ultrasound usually reveals dilated veins less than 6 mm in diameter, dilated arcuate veins connecting with bilateral pelvic varicose veins around the myometrium, and slowed and even inverted blood flow in the ovarian veins (56).

Pelvic varicosities show up as dilated, tortuous structures in the uterine adnexa on CT and MR imaging. Additionally, MR and CT imaging can detect coexisting pathology, such as tumors, and offer a thorough examination of the pelvic anatomy. The varicosities linked to PCS are highlighted using a variety of MR imaging sequences, such as gradient echo sequences that exhibit high signal intensity within the ovarian and pelvic varices (57). An ovarian vein diameter of at least 8 mm or at least four ipsilateral pelvic veins of varying caliber and at least one measuring at least 4 mm in maximum diameter is required for the diagnosis of pelvic varices using cross-sectional CT and MR imaging (57) Despite these criteria, the significance of pelvic varices on these imaging tools is evaluated subjectively. Due to its superior functional imaging and lack of radiation exposure, contrast-enhanced MR imaging may eventually replace CT as the initial imaging study for the diagnosis of pelvic venous inadequacy. Time-resolved MR angiography has been established as a reliable, noninvasive method for determining venous reflux. Time-resolved MR angiography and conventional venography were compared in a study for ovarian venous reflux, and the results showed no discernible differences between the two methods (58).

The gold standard for diagnosing this pathology consisting of venous dilatation and venous reflux as well as scheduling embolization therapy is venography, which has long been the case. By directing a catheter from the peripheral veins such as jugular, brachial, or femoral veins to the ovarian or

internal iliac veins and administering a contrast agent, catheter-directed venography is performed. Ovarian vein diameter greater than 10 millimeters, congested vulvovaginal, pelvic, or upper leg veins, and retrograde filling are venographic diagnostic indicators of pelvic venous incompetence (59) The diagnosis of PCS may also be supported by venography performed using a catheter placed in the distal left renal vein, which may show reflux of contrast material to the left ovarian vein. An important benefit of venography is the ability to administer interventional treatment if necessary, in addition to providing fantastic visualization of incompetent pelvic veins (58,59).

DIFFERENTIAL DIAGNOSIS

There are several alternative diagnoses for pelvic congestion syndrome. It encompasses illnesses of the urogenital system, digestive system, musculoskeletal system, neurological disorders, gynecological issues, and mental health issues. The most prevalent causes of chronic pelvic pain include interstitial cystitis, pelvic inflammatory disease, myalgia, endometriosis, irritable bowel syndrome with either diarrhea or constipation, and myofascial ache of the pelvic floor. Even with the use of laparoscopic and diagnostic radiological tests, it might be difficult to determine the exact underlying cause of chronic pelvic discomfort (60).

TREATMENT

Due to a lack of information about long-term efficacy, pharmacological treatment options for PCS are limited (46). Gonadotropin-releasing hormone (GnRH) agonists and medroxyprogesterone acetate (MPA) are two examples of hormone treatments that inhibit ovarian function, but they have several side effects. Additionally, only in conjunction with psychotherapy were stable outcomes obtained 9 months after MPA treatment (61). Although not curative, nonsteroidal anti inflammatory drugs (NSAIDs) might lessen symptoms while the patient receives additional treatment (47).

Micronized purified flavonoid fraction (MPFF) is a venoactive agent that has been shown to lessen the severity of pelvic symptoms brought on by pelvic varicose veins, including heaviness, pain, and labia majora swelling. These symptoms included swelling, pain, and labia majora discomfort (62). Furthermore, a double dose of MPFF (1000 mg twice daily) promotes a faster resolution of symptoms (63).

In a study by Gavrilov et al., 81.3% of patients experienced a reduction in dyspareunia, chronic pelvic pain, and discomfort after wearing class II compression shorts for two weeks. They also lessened swelling and weight

on the legs. Unfortunately, they had no impact on the vulvar varicose veins. In the group wearing elastic stockings, no clinical or venous drainage improvement was seen (64).

Extraperitoneal resection applied on the left ovarian vein was the first surgical procedure to be used to treat PCS, done by Rundqvist et al. in 1984 (65). However, compared to endovascular therapy, surgery is linked to a lengthier hospital stay and higher mortality (5).In 1993, Edwards (66) published the first representation of embolization as a treatment for PCS. Embolization therapy is advised as a 2B level of evidence, according to the Society for Vascular Surgery and American Venous Forum, for the treatment of PCS (67).

There is no established endovascular treatment protocol for PCS due to the lack of awareness of this disease. Publications on this subject use a variety of techniques, such as intravascular access sites, and embolization materials, such as sclerosants, coils, and plugs (68).

According to various studies, sustained clinical improvement after embolization ranges between 47 and 100% (69). Within the first three months, symptoms typically improved 75% of the time on average (70). Better clinical improvement may be linked to ovarian veins with smaller diameters (71).

How many veins should be embolised is still up for debate (72). When unilateral and bilateral embolizations are compared, the difference is not statistically significant (73). Some medical professionals only complete unilateral ovarian vein embolization, while others do full embolization. With a 5-year follow-up following the procedure, Laborda et al. reported the outcomes of coil embolization of both hypogastric veins and both gonadal veins in PCS patients. Clinical progress was made in 93.85% of cases (74). Because the branches of the IIVs and the ovarian veins are connected, it would appear that all insufficient venous outlets should be closed and that each trunk should be embolised when the ovarian vein multiplies. Embolization can also be performed with CO2. Because CO2 has a lower density than the tissue around it, it is categorized as a negative contrast agent. It is a must to use equipment with the right software tailored to negative contrast media to image the vessels with CO2 contrast. The biggest benefit of CO2 is its lack of side effects that are typically associated with iodine-containing substances, particularly allergic reactions, and kidney damage. However, when administering CO2, the patient may feel pain or discomfort, and the vein may respond by spasming (75).

Removal of the obstruction is necessary when stenosis is present and poses a significant hemodynamic risk (76). Stent occlusion poses the greatest risk of treatment failure, and different studies have found different

lengths of post-procedure antithrombotic therapy (77). CIV stent patency rates ranged between 60% and 100% according to a systematic review by Padrnos et al. (78). In the management of the NCS, left renal vein stenting has shown some promise in the treatment of PCS brought on by this pathology. Applying stent procedure to left renal vein carries a significant risk of relocation of the stent to the vena cava and even heart because of its length being short and tendency to change in size especially when the patient performs the Valsalva maneuver or changes positions (79). The transposition of the left renal vein might fail and is associated with grave side effects like kidney damage, bleeding, thrombosis, or infection (80,81). Percutaneous embolization complications are typically infrequent and harmless. Recurrence of the pain, a hematoma at the site, an allergic reaction, the migration of an embolic agent, or coil erosion is some of these (82).

After ovarian vein embolization, PCS symptoms may return from other venous network reservoirs. Recurrence of PCS symptoms four years after embolization was reported by Hasjim et al. Even though the gonadal vein was still embolised, the median sacral vein was used for the recurrence. The symptoms disappeared as a result of coil embolization of the dysfunctional median sacral vein (83).

Twenty percent of patients may experience postembolization syndrome. Hyperthermia, increased pelvic ache, and tenderness around the area of the embolised vein are its defining features, and it typically goes away with NSAIDs (84).

CONCLUSION

Pelvic venous disorders present clinically in a variety of ways. A significant portion of cases of Pelvic Congestion Syndrome go undiagnosed, and symptoms reported by patients are frequently misjudged as a result of a lack of understanding of the condition. It is a significant contributor to female patients' chronic pelvic pain. Additionally, it may only exhibit superficial varicose veins, or it may also be accompanied by pain. There are times when symptoms are vague and hard to distinguish from those of other illnesses. Because of the PCS's diversity, some diagnoses can be very difficult. It is difficult to identify which patients have PCS symptoms, but it is crucial to do so to implement effective and targeted treatment. To fairly assess results, a standardized algorithm for experiments aimed at understanding the mechanisms causing all these symptoms would be especially beneficial.

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CHAPTER 7

BIOGENESIS AND FUNCTION OF EXTRACELLULAR CIRCULATING MicroRNAs

Serap ÖZER YAMAN[1]

1 Dr. PhD, serapozer@ktu.edu.tr, Department of Medical Biochemistry, Graduate School of Health Sciences, Karadeniz Technical University, Trabzon, Turkey. ORCID ID: 0000-0002-5089-0836

Introduction

All eukaryotic cell types express microRNAs (miRNAs), a subclass of short noncoding RNAs that are involved in post-transcriptional control of gene expression. The 3-UTR of target mRNAs is where miRNAs bind to exert their effects, controlling the stability and protein production of the targeted mRNAs (Ambros, 2004; Bartel, 2004). Human miRNAs come in more than 2812 distinct subtypes, and this number is increasing (http://www.mirbase.org/). Endogenous miRNAs in humans regulate at least 30% of their genes associated (Lewis et al., 2005). As a result, they contribute in coordinating fundamental biological functions such cell division, DNA repair, differentiation, metabolism, and apoptosis (Ambros, 2004; Bartel, 2004; Croce et al., 2005). Many diseases, including cancer, have continuously been related to the liberation of several miRNAs' expression in cells (Lu et al., 2005). Each miRNA has a distinct expression pattern in a particular cell type and a distinct nucleotide sequence (Lu et al., 2005; Landgraf et al., 2007).

All biological fluids, including blood plasma, urine, tears, breast milk, amniotic fluid, cerebrospinal fluid, saliva, and semen, were found to contain large amounts of miRNA a few years ago (Turchinovich et al., 2012). These extracellular (EC) circulating miRNAs are unexpectedly resilient, surviving harsh physiologic circumstances like boiling, numerous freeze-thaw cycles, significant pH fluctuations, and extended storage. Common RNAs, including as mRNA, rRNA, and tRNA, are quickly broken down in the nuclease-rich EC media in contrast to miRNAs. (Chen et al., 2008; Turchinovich et al., 2011). Exosomes were found to transport intracellular miRNAs into the EC environment when cells were cultured. (Valadi et al., 2007).

Gene regulation and intracellular miRNA production

Normally, transcription of cell genes results in the production of miRNA. These steps are part of the miRNA biogenesis pathway in animal cells. In the nucleus, stem-loop structures are found in primary transcripts (pri-miRNAs), which are how genes are first translated. The endonuclease Drosha will then cut the stem loop structure in the pri-miRNA, producing precursor miRNAs (pre-miRNAs) that are around 70 nt in length. After then, the pre-miRNA is moved outside of the nucleus. Exportin-5 transports it to the cytoplasm, where Dicer/TRBP (TAR RNA-binding protein) further cleaves the "loop" construct to create the mature miRNA/miRNA* duplex. Once the duplex miRNA* helix relaxes, sheds, and degrades, the mature miRNA is subsequently loaded into Argonautes (Ago) to create core effector complexes called miRNA-induced silencing complexes (miRISCs) (Iwakawa et al., 2021). The biological function of miRNA in

plants is distinct from that in animals. Due to a lack of Drosha homologue proteins, Dicer-like protein (DCL) cleaves pri-miRNA in plants, producing pre-miRNAs of hundreds of nt in length (Liang et al., 2014). By chance, a protein similar to Dicer also cleaves premiRNA, and both pri-miRNA and pre-miRNA are broken down in the nucleus. Hua enhancer 1 (HEN1) methylates the 3' terminal riboses of the plant miRNA after it is transported to the cytoplasm, ensuring its stability against strong acid, strong alkali, and high temperature (Yu et al., 2005). MiRNAs bind target mRNA sequences in animal or human cells primarily through conventional base pairing between the corresponding sequence found in the 3' untranslated region (3'UTR) and the seed sequence, which is composed of nucleotides 2 to 8 from the 5' end. As a result, the target mRNA is inhibited from transcription (Fabian et al., 2011), cleaved (Yekta et al., 2004), or degraded (Wu et al., 2006). In-depth analysis of the target mRNA, MiRNA, revealed that it also targets binding to the 5'UTR and open reading frame (ORF) to determining the importance of gene regulation (Jopling et al., 2008; Orom et al., 2008). Plant miRNAs penetrate animal or human cells and, like animal miRNAs, exercise their actions through base complement coupling with target gene mRNA (Cavalieri et al., 2016; Chin et al., 2016; Hou et al., 2018). MiRNAs are a significant class of gene regulatory molecules that regulate many features of cellular behavior, including cell growth, division, differentiation, proliferation, apoptosis, and metabolism. It is believed that miRNAs control around one-third of human gene expression (Chen et al., 2006; Lewis et al., 2005; Wu et al., 2022).

Extracellular miRNA

Many different research teams independently observed for the first time in 2008 that mature miRNAs are also include in cell-free blood plasma and serum (Chen et al., 2008; Chim et al., 2008; Lawrie et al., 2008; Mitchell et al., 2008). Then, it was established that EC circulating miRNA was present in all other bodily fluids (Park et al., 2009; Hanke et al., 2010; Kosaka et al., 2010b; Weber et al., 2010). It has been known for some time that miRNAs are present in the exosomes that cells in culture export, but the mechanism underlying their resistance to EC nucleases has remained a mystery (Valadi et al., 2007). Hunter et al. discovered miRNAs in peripheral blood microvesicles (MVs) and proposed the idea that ECmiRNA is preserved by encapsulation within membrane-vesicles (Hunter et al., 2008). They discovered proof that exosome-mediated transport can facilitate miRNA exchange between cells (Valadi et al., 2007). MVs mediate the body's organ communication system via miRNAs that are encapsulated (Hunter et al., 2008). In 2011, it was discovered by two different research teams that 90-99% of ECmiRNA is MV-free and both are linked to pro-

teins of the AGO family in blood plasma, based on the hypothesis that only membrane-vesicles-encapsulated miRNAs are present in biological fluids was completed. (Turchinovich et al., 2011; Arroyo et al., 2011). The related miRNAs' resistance in nucleases containing biological fluids was elegantly explained by the exceptional stability of the AGO2 protein in protease-rich media (Turchinovich et al. 2011).

Origin of extracellular miRNAs

It has been suggested that ECmiRNAs can be produced by blood cells and other organs (Turchinovich et al., 2013). Circulating miRNA biomarkers (approximately 79) for solid tumors that have been published in the literature, 58% of them are significantly expressed in at least one kind of blood cell. Moreover, they demonstrated a strong correlation between the levels of plasma miRNA biomarkers and the corresponding blood cell counts or hemolysis, indicating that serum/plasma miRNAs are mostly sourced from blood cells (Pritchard et al., 2012). Plasma has also been found to contain certain tissue-enriched miRNAs, such as liver-enriched miRNA-122, muscle-enriched miRNA-133, heart-enriched miRNA-208, and brain-enriched miRNA-124 (Laterza et al., 2009; Corsten et al., 2010; Lewis et al., 2010; Zhang et al., 2010). Three separate mechanisms each allow for the release of such miRNAs into the EC environment: (1) passive leaking from damaged cells brought on by tissue injury, inflammation, cell necrosis, or apoptosis. (2) Active secretion through membrane-enclosed cell fragments known as MVs, which are secreted by nearly all cell types under healthy and pathological situations and include vesicles and exosomes (Cocucci el al., 2009; Théry et al., 2002; Mathivanan et al., 2010; Ratajczak et al., 2006; Simons et al., 2009).. (3) RNA-binding protein-bound route for active secretion without MV. Several RNA-binding proteins, including as high-density lipoprotein (HDL) (Vickers et al., 2011), Argonaute 2 (AGO2) (Arroyo et al., 2011; Turchinovich et al., 2011) and nucleophosmin 1 (NPM1) (Wang et al., 2010) have been shown to interact with and transmit miRNAs outside of cells. Secreting miRNAs via MVs and HDL binding is active and requires energy compared to passive leaking. It is believed that ECmiRNAs released by active pathways regulate biological activities (Zhao et al., 2019).

Connection between Cells through External Cell MiRNA

The concept that cells deliberately deliver miRNAs that regulate cell-cell signaling via paracrine and even endocrine pathways was sparked by the discovery of miRNA in the EC environment (Valadi et al., 2007; Cortez et al., 2011; Chen et al., 2012). Nevertheless, nonspecific residues originating from cell physiological activity and cell death bind circulating miR-

NAs only by AGO proteins (Turchinovich et al., 2011). As a result, long after the original cells have died, both the AGO2 protein and miRNAs stay stable. In mammals, there is also no evidence that AGO-miRNA ribonucleoprotein complexes are actively discharged from cells or taken up by recipient cells. The idea that many ECmiRNAs are released non-selectively following cell death is also in line with the observation that toxicity in some tissues raises the level of tissue-specific miRNAs in blood. (Laterza et al., 2009; Corsten et al., 2010, Lewis et al., 2010; Zhang et al., 2010; Pritchard et al., 2012). Meanwhile, numerous independent studies have demonstrated that ECmiRNAs contained in apoptotic bodies and exosomes can be transported to recipient cells, change gene expression, and have functional effects (Valadi et al., 2007; Skog et al., 2008; Turchinovich et al., 2013).

Extracellular miRNA carriers

Blood samples were found to contain EC short RNAs that were non-coding small RNAs (El-Hefnawy et al., 2004). Although Lawrie et al. reported finding ECmiRNAs in lymphoma patients' serum; Mitchell et al. discovered that ECmiRNAs are persistent in human plasma/serum (Lawrie et al., 2008; Mitchell et al., 2008).

Surprisingly, more than ten years ago, miRNAs were shown to be useful EC signaling molecules in plants (Baulcombe, 1996; Voinnet et al., 1997). Global plasmid miRNAs, on the other hand, are made up of many subclasses of miRNA transporters. ECmiRNAs have been discovered to be transported through ribonucleoprotein complexes, lipoproteins, and membrane-derived vesicles (Valadi et al., 2007; Zhang et al., 2010; Vickers et al., 2011). Exosomes and microparticles (MPs) are two distinct kinds of membrane-derived vesicles that can be distinguished from one another by their secretory and biogenic processes. Endosome-containing bodies at the fusion of endosomes with the plasma membrane, exosomes and their miRNA contents are released into the EC compartment (Théry , 2011). In contrast, MPs are bigger (100–4000 nm) vesicles that typically form via bursting and branching outward (Mause et al., 2010). As cells undergo plasma membrane apoptosis, they may expel much larger MPs or apoptotic bodies that also contain specific miRNA sets (Zernecke et al., 2011).

Whereas lipoproteins have a single lipid layer, a hydrophobic core, and are defined by certain structure and function apolipoproteins, exosomes and MPs have a bilayer phospholipid shell and hydrophilic center. Although some miRNAs, like miRNA-223, are present in all subclasses, there is evidence that the exosomal, HDL, and LDL-miRNA signatures differ (poor correlation) (Vickers et al., 2011). Both inside and outside of the membrane-derived vesicles, Argonaute 2 (AGO2), the primary useful component of the cytoplasmic cell, the miRNA ribonucleoprotein complex

(miRNP), is seen coupled to external miRNAs. According to biophysical studies, miRNAs are connected to protein complexes that range in size from 50 to 300 kDa and contain ribonucleoproteins such AGO2. Recently, it has been discovered that viral surface antigen particles carry certain miRNAs in addition to hepatitis B surface antigen particles (Collino et al., 2010; Arroyo et l., 2011; Turchinovich et al., 2011).

Most cell types, including neurons, inflammatory, muscle, and tumor cells, release exosomes or MPs, according to in vitro investigations (Valadi et al., 2007; Skoget al., 2008, Rosell et al., 2009). Nevertheless, platelets are likely responsible for the majority of circulating MPs and exosomes in vivo (VanWijk et al., 2003; Boon et al., 2013). Many different miRNA profiles connected to membrane-derived vesicles have been reported. Whether individual miRNAs carried by external protein complexes vary in quantity as a result of disease is currently unknown. Similar to this, distinct HDL-miRNA signatures have been found in hypercholesterolemic mice and humans (Vickers et al ., 2011). Most intriguingly, miRNA-150, miRNA-223, and miRNA-92, which make up the majority of the differential miRNAs linked to cardiovascular illness, have all been found to change gene expression when transferred to recipient cells. Hence, they are potential signal molecules (Zhang et al., 2010; Vickers et al., 2011; Umezu et al., 2013).

Cellular miRNA Export

Intercellular communication based on miRNAs involves three crucial steps. MiRNAs must first be actively and selectively released from cells into the proper carriers. The transfer of miRNAs to recipient cells with specified targets or receptors requires two additional steps. The third and most crucial requirement is that miRNAs continue to recognize and inhibit mRNA targets in recipient cells. Since some miRNAs are only exported and not retained in the host cell, and since specific signaling pathways have been discovered to control cellular miRNA release, there is a lot of evidence to suggest that the process is selective and regulated, even though the exact mechanism by which miRNAs are selectively exported is still poorly understood (Wang et al., 2010; Vickers et al., 2011; Umezu et al., 2013). Most importantly, the miRNA profiles of lipoproteins and EC vesicles are separate clusters of miRNAs rather than major cell types (Valadi et al., 2007; Zhang et al., 2010). Moreover, several miRNA fingerprints are present in each compartment of biological fluid. All of these findings are consistent with the selective export theory, which states that certain miRNAs are actively secreted by cells in response to signals from other cells or environmental factors. It was shown that EC vesicles released from tumor cells included miRNAs that were absent from the parent cell, indicating that some miRNAs can only be

exported (Ohshima et al., 2010; Pigati et al., 2010). It is either transcribed or processed in a specific cell, but EC vesicles may distribute it exogenously to other cells. The ceramide route controls the export of cellular miRNAs according to numerous studies (Wang et al., 2010; Vickers et al., 2011). The rate-limiting enzyme in the transformation of sphingomyelin to ceramide, neutral sphingomyelinase 2 (nSMase2), is a crucial regulator of exosome biogenesis and secretion (Trajkovic et al., 2008). The export of certain miRNAs to exosomes was decreased when nSMase2 was inhibited, but not the export of miRNA-223 to HDL (Trajkovic et al., 2008; Boon et al., 2013). As miRNA-223 is one of the most prevalent and consistent miRNAs detected in HDL, it has previously been discovered to be rich in non-vesicle subtypes (Zhang et al., 2010).

Transport of Essential miRNAs

Apoptotic bodies were discovered to transport functional miRNA-126 to recipient cells in 2009, marking the beginning of endogenous transfer of functional miRNAs (Zernecke et al., 2009). In 2010, exosomes with functional miRNAs were discovered to be transported into mother cells that were Epstein-Barr virus-infected. Exosomes harboring viral miRNAs have been shown to be secreted, and these exosomes can change target genes in recipient dendritic cells Pegtel et al., 2010). Exogenous miRNA targeting and endogenous miRNA gene regulation were easily distinguished (Meckes et al., 2010; Pegtel et al., 2010). These discoveries most critically presented the idea of miRNA intercellular communication. Parallel to this, MPs were discovered to promote migration by transferring miRNA-150 to recipient endothelium cells (Zhang et al., 2010). A unique miRNA-based transmission network between inflammatory cells and the vascular endothelium was discovered by this study and studies that came after it. EC has been proposed as the explanation. In a microenvironment, miRNAs are particularly skilled in mediating gene regulation between cells. In this situation, a miRNA-based link may exist between sub-intimal inflammation and atherosclerotic lesions that contain intricate multicellular microenvironments (Boon et al., 2013).

Endothelial cells have been discovered to be highly effective recipient cells for miRNA transfer in numerous studies (Zhang et al., 2010; Wang et al., 2010; Umezu et al., 2012). Yet, a recent study discovered that activated endothelial cells produce vesicles that transmit miRNA, indicating that endothelial cells also emit ECmiRNAs.

Cellular to Cell Communication

Although though cell-to-cell miRNA transport may be better suited for microenvironments, lipoproteins and EC vesicles are found in plasma

and may go to a variety of distant organs. Several other pathophysiologies, excluding cardiovascular disease, probably use circulating transporters to alter systemic homeostasis or spread illness. Circulating miRNAs and their transporters are likely used by cancer to promote tumorigenesis and malignancy, according to the evidence (Umezu et al., 2012; Zhuang et al., 2012). Malignancy is caused by the interaction of tumor cells with endothelial cells through cell migration, angiogenesis, tumor growth, and the transfer of pro-angiogenic miRNAs (Zhuang et al., 2012). MiRNAs that regulate carcinogenesis and cellular invasiveness at birth have also been discovered in other research to be released by tumor cells (Umezu et al., 2012). Recently, it was discovered that miRNAs in exosomes activate Toll-like receptors in endosomes, causing the recipient to develop oncogenic characteristics (Boon et al., 2013).

Adipocytes can communicate both locally and systemically by using miRNAs. Little adipocytes get miRNA-16, miRNA-222, miRNA-27a, and miRNA-146b from large adipocytes, which promote lipid synthesis (Müller et al., 2011). Due to diverse stressors, miRNA-induced lipid storage may not be close preserved and may function as a potent metabolic signal. Adipocytes and fat can also connect with inflammatory cells through the transfer of miRNA (Ogawa et al., 2010). Most intriguingly, mothers likely release exosomes into the maternal circulation via chronic villi to transfer miRNAs to their unborn children in the womb. Furthermore, it has been discovered that fibroblasts receive miRNAs from embryonic stem cells (Yuan et al., 2009).

MiRNAs' role in digestion and associated processes

Exogenous miRNAs, such as plant miRNAs, are absorbed into the bloodstream through the digestive system and are a significant source of circulating miRNAs. In 2011, studies discovered that roughly 5% of the miRNAs in human and animal blood have anti-sodium periodate capabilities. Based on this discovery, they hypothesized that plant miRNAs consumed daily may be their likely source and supported their hypothesis with evidence from food. It enters the bloodstream of the body through the digestive tract. The study team then verified through studies that liver low-density lipoprotein receptor adapter protein 1 (LDLRAP1) is concurrently targeted and inhibited by mouse miRNA-168a, decreasing its expression and ultimately increasing low-density lipoprotein (LDL) metabolism (Zhang et al., 2012). Since then, more investigations have consistently supported the idea that exogenous plant miRNAs can enter the bloodstream through the digestive system and travel to distant tissues and organs in the body. After mice were given ice-cold cabbage, cabbage miRNA was found in their intestines, blood, spleen, liver, kidney, feces, and other samples

(Liang et al., 2014). When subjects consumed watermelon juice or a combination of fruits, Liang et al. found a variety of matched fruit miRNAs in their plasma (Liang et al. 2015). Pigs fed fresh maize for 7 days had serum, pancreas, and longissimus dorsi that contained 16 maize miRNAs (Luo et al. 2017). Moreover, Tarallo et al. discovered that there are notable variations in the fecal miRNA expression profiles of vegans, vegetarians, and omnivores, suggesting perhaps the impact of dietary miRNA on the human body (Tarallo et al., 2022). According to the aforementioned justification and supporting data, the researchers looked deeper into how herbal miRNA was absorbed by the body and discovered that it was still able to exist steadily even after boiling at a high temperature and could enter the bloodstream through digestion (Li et al., 2015; Zhou et al., 2015; Kalarikkal et al., 2021; Teng et al., 2021). In addition to plant miRNAs, miRNAs in milk exosomes can also be ingested through the digestive system and taken into the blood (Manca et al. 2018). The in vitro injection of circulating miRNA into our body makes sense for its practical application. (Teng et al. 2021).

The potential of some plant or herbal miRNAs to resist high temperatures while being cooked, ground, digested, or degraded has been demonstrated by recent study. The following elements can be considered the causes: (1) Plant miRNAs' unique sequence composition and/or 3' end methylation (Zhou et al., 2015); (2) Strong heat and acid stability in plant cell exosomes allows them to successfully protect the miRNAs they have encapsulated (Lasser et al., 2011; Mu et al., 2014). Different plant miRNA expression profiles in food and plasma suggest that the body also absorbs plant miRNAs in a targeted manner (Zhang et al. 2010). Predicted miRNA forms in food contain pri-miRNA, pre-miRNA, miRNA:miRNA*duplex, free miRNA, AGO2-linked miRNA, and miRISC based on the synthesis and activity of miRNA. Researches have demonstrated that mature free miRNAs can enter the bloodstream and become circulating miRNAs (Zhang et al., 2012; Chin et al., 2016; Hou et al., 2018). Exogenous plant penetration may be mediated by two different ways. MiRNAs circulate throughout the body. Gastrointestinal epithelial cells actively produce plant miRNAs into the blood after they have been first absorbed by them (Jia et al., 2021). SID-1 transmembrane family member 1 (SIDT1) in the plasma membrane of stomach epithelial mucus cells has been demonstrated to have the ability to transport dietary plant miRNAs into the blood in both in vitro and in vivo investigations (Chen et al. 2021). Second, plant miRNAs are contained in plant exosomes and taken up by gastrointestinal epithelial cells through endocytosis; after this, gastrointestinal epithelial cells actively secrete the miRNAs into the blood (Kusuma et al., 2016; Manca et al., 2018).

Function of Extracellular miRNAs in Disease

When cells are stressed or exposed to metabolic stimuli, cellular miRNAs have been shown to be effective mediators. To completely comprehend the part ECmiRNAs play in both adaptive and maladaptive responses to disease, however, further research is required. There is evidence for consistent ECmiRNA signatures in health, which may represent a novel metric for assessing health and identifying disease. Although each subclass of miRNA transporters likely has a unique miRNA profile in both health and sickness, some miRNAs, like miRNA-223, which is present in both exosomes and HDL, are strictly shared by all carriers. As a result, miRNAs may be transported or exchanged across carriers of both comparable and disparate types. The physical transfer of miRNAs into the recipient cells' cytoplasm or into miRNA complexes raises another unanswered query about the cell-to-cell transfer theory (Vickers et al., 2011). For the distribution of HDL cholesterol ester, the HDL receptor SR-BI employs a selective core uptake method. SR-BI expression and function are necessary for HDL-miRNA delivery; however it is still unknown whether selective core recruitment is different from the miRNA delivery pathway (Acton et al., 1996).

As with SR-BI, it is unclear whether LDL receptor-mediated endocytosis transports functional LDL miRNAs to recipient cells or whether SR-BI facilitates the reception of LDL-miRNAs (Acton et al., 1996). MiRNAs have been seen to be transferred through membrane fusion and endocytosis in membrane-derived vesicles. Yet, miRNAs taken up via endocytosis still need to traverse a phospholipid bilayer in order to enter the cytosol for target recognition and functionality. Membrane fusion is expected to release miRNAs straight into the cytosol (Morelli et al., 2004., Tian et al., 2010). As a result, there are presumably many proteins that function as sensors, transporters, and transporters inside the cell membrane of endosomes and lysosomes that are currently unclear.

Targeting extracellular miRNAs for treatment

Certain genes can be targeted by miRNAs and siRNAs, and protein expression levels can be controlled. These RNA molecules so appear potential treatments for a number of diseases by modifying disease genes that are aberrantly expressed (Ryther et al., 2005). The effective and precise distribution of short RNAs is essential for their successful use in clinical practice (Weiler et al., 2006). Because of rejection reactions, conventional short RNA delivery methods involving viruses and liposomes are hazardous and ineffective. MVs, on the other hand, can naturally interact with plasma membranes and carry packaged functional short RNAs into cells in a way that is better tolerated by the immune system since they are

membranous vesicles that are released by practically all cell types. As a result, MVs may one day serve as a safe and effective means of delivering therapeutic RNAs. MVs may be able to deliver drug siRNAs to certain target cells for therapeutic effects, according to some research. Exosomes made from dendritic cells (DCs) expressing a fusion of the rabies virus glycoprotein (RVG) peptide and an exosomal membrane protein (Lamp2b) were synthesized by Alvarez-Erviti et al. and intravenously injected into mice (Alvarez-Erviti et al., 2011). In mouse brains, they discovered that packaged siRNA in these RVG-targeted exosomes may be directed into neurons, oligodendrocytes, and microglia, leading to the silencing of a particular gene. Also, they put siRNA of BACE1, a therapeutic target for Alzheimer's disease, into exosomes that were RVG-targeted and administered them intravenously to wild-type mice. BACE1 was significantly downregulated in both mRNA and protein by the results in mouse brains, which points to the therapeutic potential of this exosome-mediated siRNA delivery mechanism (Alvarez-Erviti et al., 2011). Additionally, using exosomes similar to RVG, our team created an additional exosome that was loaded with the opioid receptor mu (MOR) siRNA. We discovered that these exosomes can effectively deliver siRNA to the mouse brain, significantly lower MOR mRNA and protein levels, and effectively prevent morphine relapse. It's interesting to note that they demonstrated the association between medication MOR siRNAs and AGO2 in RVG exosomes (Liu et al., 2015). Together, findings show that exosomes have the ability to deliver tiny RNAs to certain destinations, and that this property of exosome-transported RNAs will open up new opportunities for therapeutic uses.

Summary

ECmiRNAs can be actively released and transported, and then they can attach to target genes in recipient cells and regulate them. ECmiRNAs are detected stably in numerous body fluids by MV encapsulation or by binding to proteins. We presently have enough data to understand the biology of ECmiRNAs and assess ECmiRNAs isolated from serum, plasma, or other bodily fluids as a possible developmental regulator and a prospective disease biomarker. Yet, the conflicting findings in the ECmiRNA study brought on by the technical and experimental setting spark a lot of debate. It is advised to take a few key factors into account before planning studies to investigate ECmiRNAs. The low concentration of miRNA in biofluids or cell culture media is one of the main problems, so it's critical to pick a platform that enables analysis of ECmiRNAs with small amounts of starting materials. Several studies are now working on techniques to extract the most total RNA possible from liquid samples for ECmiRNA profiling. The type of ECmiRNAs under investigation—exosomes, MVs, HDL, or

protein-associated miRNAs—is another crucial factor. Many aspects of the released miRNAs' intracellular sorting mechanisms remain unclear. Further research is needed to determine whether other patterns are connected to the packing of specific miRNAs into shed vesicles or how they bind with HDL. As many transcripts might be coupled with a miRNA in recipient cells, it is also necessary to determine how donor cells control their secreted miRNAs to target particular genes. Exosome-derived miRNAs are challenging to precisely quantify because to variations in measurement techniques and random disruption. The therapeutic effects of ECmiRNAs may also be impacted by a number of delivery-related obstacles, including poor bioavailability, low tissue permeability, and inadequate transport capacity of circulating miRNA antagomirs or mimics. As not all ECmiRNA release and transport mechanisms are biologically feasible, it may be more advantageous to concentrate on specific ECmiRNA types, such as exosomal miRNAs or protein-linked miRNAs, rather than the overall number of ECmiRNAs present in a biological fluid. In fact, as a young field, ECmiRNA research still needs major technological advancement. ECmiRNAs have also been studied for their potential clinical uses as diagnostic biomarkers and therapeutic agents. ECmiRNAs have also been studied for their potential clinical uses as diagnostic biomarkers and therapeutic agents. Very fascinating are the recent ECmiRNA discoveries. Thus, it is crucial to investigate more precise quantitative techniques and effective ECmiRNA delivery routes in further investigations. Nonetheless, this new topic might deepen our comprehension of intercellular and human communication. For ECmiRNA research to produce accurate results, a systematic procedure must be created. Understanding ECmiRNAs will be crucial to improving our understanding. The constraints of ECmiRNA research will undoubtedly be addressed in the near future, and this will considerably advance our knowledge of the biology of development and disease and may even pave the way for the creation of novel treatments.

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CHAPTER 8

EFFECTS OF LONG NONCODING RNAs ON ORAL SQUAMOUS CELL CARCINOMA

Serbülent YİĞİT[1], Serkan YILDIZ[2], Melih Sercan USTAOĞLU[3]

1 Doç.Dr. Department of Genetics, Faculty of Veterinary Medicine, Ondokuz Mayıs University, Samsun, Turkiye ORCID: 0000-0002-1019-3964
2 Dr. Öğr. Üyesi Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Istanbul Aydin University,Istanbul, Turkiye ORCID: 0000-0002-5588-9367
3 Öğr. Gör. Amasya University Suluova Vocational School Veterinary Department ORCID: 0000-0001-9380-446X

Introduction

Head and neck squamous cell carcinoma (HNSCC), originating from the oral cavity, pharynx, and larynx mucosal epithelium, constitutes approximately 2.4% of all malignant tumors. It is the sixth most common malignancy in developed countries (1). The most common type of HNSCC is oral squamous cell carcinoma (OSCC) (> 90%) (2). Worldwide, approximately 600,000 new cases occur annually. These are too often seen in developing countries, especially in Asia (3). These are 5% of all cancers in men and 2% of all cancers in women (4). Examination of the characteristics of OSCC showed that tumor stage or grade did not differ with age (5). Although early screening and individualized treatment options have improved, OSCC still maintains its importance.

Cancer formation is a multi-stage process that occurs with the accumulation of genetic and epigenetic changes in cancer-related genes over time (6). OSCC is caused by a lot of genetic changes caused by many factors. Recurrent and metastatic OSCC often cannot be cured. In addition, our knowledge about the clinical course, biology, and genetic biomarkers of this disease is not fully sufficient. Despite the improvement of diagnosis and treatment methods, the 5-year survival rate of patients has not changed and is still below 50% (7). Therefore, understanding the molecular mechanisms of the disease is important for early diagnosis, appropriate treatment, and prognosis. Today, cancer treatment is evolving to be targeted specifically at the individual. The most widely used treatment is targeted immunotherapy, which improves 5-year survival in many cancers (8). In this review, we will summarize the role of long non-coding RNAs (lncRNAs) in OSCC.

Risk factors for oral cancer

Oral cancer is a critical healthcare problem. It is counted as the main cause of death from oral diseases in many countries. OSCC is more common in underdeveloped countries. Risk factors for OSCC include carcinogens that may synergize during oral carcinogenesis. It is estimated that 75% of all oral cancers are caused by preventable causes, since relative risk factors such as alcohol and tobacco are at the forefront. Apart from these, the cause of cancer is not completely clear in the other 25% (9). The higher incidence of head and neck cancer in some countries than other malignancies may be due to low socioeconomic status associated with tobacco and alcohol consumption, poor hygiene, malnutrition, and widespread viral infections (10).

Tobocco: In Asian countries, especially India, oral cancer is thought to be associated with betel quid (BQ) and chewing tobacco. In westbound

countries, smoking and excessive alcohol consumption are important danger factors (11). The international authoritative cancer agency has confirmed that various types of tobacco are carcinogenic to humans (12). Chewing BQ-containing tobacco increases exposure to tobacco-specific toxins. In addition, the production of significant amounts of reactive oxygen species in the oral cavity during chewing is also a factor in the development of cancer.

Betel quid and Areca nut: Betel quid (BQ) is a toxic agent for health and has a carcinogenic effect. With concurrent use of tobacco, alcohol, and betel, the risk of developing oral cancer increases 123 times (13). Chewing BQ produces ROS that induce mutation or sensitize the mucosa to environmental toxicants, leading to tumor initiation.

Alcohol: Alcohol has the ability to increase the permeability of the oral epithelium. It acts as a solvent for tobocco contents. It increases the production of free radicals and acetaldehyde that cause DNA damage (14). In oral carcinogenesis, alcohol acts independently and synergistically with smoking (12). In addition, it can increase the penetration of carcinogenic substances into target tissues with its solvent function. Acetaldehyde, a metabolite of alcohol, has been reported to be a tumor promoter (12).

Socioeconomic conditions: It is worse in those with low socioeconomic status due to poor oral hygiene and difficulty accessing medical controls (15).

Viruses: The human papillomavirus (HPV) family contains more than 170 different types of viruses that are capable of infecting stratified epithelium. HPV infection is an independent etiological factor for OSCC. HPV, which is associated with benign and malignant oral lesions, is observed in various oral lesions. A minority of HPV-infected lesions, especially those with HPV 16 and 18 subtypes, rarely turn into malignancy (12).

OSCC genetics

Carcinogenesis develops with multiple genetic events that occur with the alteration of the normal functions of oncogenes and tumor suppressor genes. As a result of pathological changes from hyperplasia to dysplasia in oral carcinogenesis, these changes end with invasion and metastases (16). Under normal conditions, the cell biology, cell division, and differentiation of the oral epithelium are controlled by stimulating and suppressive pathways (17). Oral cancer occurs with the accumulation of changes in these excitatory and inhibitory signals that may occur at any level of a particular pathway. Tumor cells begin to spread to local or distant sites with rapid division and the formation of new blood vessels (18).

Long non-coding RNAs

Advances in next-generation sequencing technology have enabled comprehensive transcriptomic and bioinformatic analysis in many cell tissues. As a result, it has been shown that although more than 75% of the human genome is transcribed, only a small fraction of the transcripts are translated into protein products (19). Transcripts without any protein-coding capacity were defined as non-coding RNAs (ncRNAs). They are divided into two groups according to their length. Small ncRNAs shorter than 200 nucleotides consist of microRNAs (miRNA) and small nucleolar RNAs (snRNA) (20).

Long non-coding RNAs (lncRNAs) longer than 200 nucleotides are synthesized by RNA polymerase II and cannot code for proteins or peptides (21). H19 was identified in a 1989 study as the first lncRNA to be highly expressed in mouse embryos and neonatal livers. The mouse H19 gene is quite similar to this human gene, but the specific function of the gene is unknown (22). For many years, lncRNAs were thought of as "junk transcripts" with no function. It has been shown that lncRNAs can interact with biological macromolecules (23). In general, lncRNA expression levels are lower than those of protein-coding genes (24). Inherited changes in gene expression without changes in the DNA sequence are called epigenetics. Epigenetic mechanisms include DNA methylation, histone modification, chromatin remodeling, and broadly non-coding RNA (25). LncRNAs regulate gene transcription at the transcriptional level by modulating histone or DNA modification through epigenetic mechanisms. It can also regulate histone methylation or acetylation alone (26). To date, there are more than 127,000 high-confidence lncRNA transcripts determined in Homo sapiens (https://lncipedia.org/).

LncRNAs in cancer

Cancer is an important life-threatening cause of morbidity and mortality. Despite the recent advances in technology, satisfactory results in terms of diagnosis, treatment, and prognosis have not been achieved (27). As a result of her studies investigating the function of lncRNAs, a relationship between cancer and lncRNAs has emerged. lncRNAs such as suppressor genes or oncogenes influence tumorigenesis and tumor development. LncRNA has been found to be abnormally expressed in various cancers such as liver cancer (28), breast cancer (29), bladder cancer (30), and colorectal cancer (31), and regulate tumor cell metastasis in unalike ways. LncRNAs can affect tumor metastasis by acting on various biological processes, such as chromatin modification (31), transcription activation (32), transcription

interference (33), RNA stability regulation (34), and mRNA translation (29).

The roles of lncRNAs in OSCC

OSCC carcinogenesis is a multifactorial and multistep process involving genes, epigenetics, and the environment (35) . Studies have shown that abnormal lncRNAs contribute to biological behavior, clinical diagnosis, prognosis, and treatment options in OSCC. HOX antisense intergenic RNA (HOTAIR) is the first identified lncRNA (36). HOTAIR expression level was found to be associated with metastasis, tumor differentiation, grade of malignancy, and prognosis. Further, upregulation of HOTAIR expression promoted OSCC cell proliferation, invasion, metastasis, and angiogenesis by binding to EZH2 and H3K27me3 and ultimately silencing the E-cadherin gene (37). It acts as an oncogene in OSCC by releasing H19 EZH2, which competes with miR-138. High expression of H19 was associated with TNM staging, lymph node metastasis, and poor prognosis (38). Bioinformatics analyses demonstrated that there are 160 differentially expressed lncRNAs between OSCC and normal control tissues. LncRNAs (FTH1P3, PDIA3F, and GTF2IRD2P1) influence the progression and metastasis of OSCC by triggering some metalloproteinases and cytokines (39). There are 21 lncRNAs that are significantly associated with overall survival and disease-free survival (40).

Surgical resection is a treatment modality used in the early stages of OSCC patients. However, recurrence after surgical resection can lead to death. Factors such as tumor location, etiological factors, clinical stage, and treatment may affect the course of the disease (41). Dong et al. showed that plasma levels of lncRNA CASC2 decreased in patients with local recurrence and increased in patients without recurrence. CASC2 can be used for the prognosis of OSCC after surgical resection as lncRNA CASC2 overexpression promotes cancer cell proliferation (42). High expression of HNF1A-AS1 in OSCC samples was associated with a poor prognosis, whereas HNF1A-AS1 deletion inhibited proliferation, migration, and EMT of OSCC cells (43). Elevated MALAT1 levels were associated with poor prognosis in 54 OSCC tumor samples (44). High LINC01133 expression was associated with fewer metastases and a better prognosis in OSCC (45).

Clinical importance of lncRNA in OSCC

Many lncRNAs have been shown to be abnormally expressed in various cancers. Some of these are more cancer-specific. Many were detectable in the plasma and urine of cancer patients as they were stable in body fluids. In addition, expression levels could provide information about the status of cancers. These suggest that lncRNAs may contribute as noninva-

sive biomarkers and therapeutic targets (46). LncRNAs differ from protein-coding genes in many ways. It is advantageous to have lncRNA-based biomarkers specific to each cancer subtype, as lncRNAs are more abundant than protein-coding genes. In addition, subtype/tissue-specific lncRNA expressions are important for the development of new diagnostic biomarkers and personalized treatments (47). Because of their diverse cellular signaling pathways and tissue-specific expression, lncRNAs can be used in the diagnosis of cancer subtypes and in the development of new strategies.

Conclusion

Studies to understand the functions and functioning of lncRNAs in cancer have increased in recent years. The use of animal models and next-generation technologies will help to understand the relationships between lncRNAs. In many studies, it is known that determining the expression profile of lncRNAs affects the molecular phenotype of the tumor. Elucidation of tumor molecular structure will be a target for new treatment strategies.

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CHAPTER 9

SIMULATION AND STANDARD PATIENT USED IN PSYCHIATRIC NURSING EDUCATION: SYSTEMATIC REVIEW

Nesibe GÜNAY MOLU[1], Birgül ÖZKAN[2]

1 Necmettin Erbakan Universty, Faculty of Nursing, Department Nursing, Konya/Turkey, E- mail: nesibegunaymolu@gmail.com, ORCID: 0000-0002-2452-6205

2 Ankara Yıldırım Beyazıt Universty, Faculty of Health Sciences, Department Nursing, Ankara/Turkey, E-mail: ozkanbirgul7@gmail.com, ORCID: 0000-0002-1271-8007

INTRODUCTION

Being one of the innovations gradually increasing in technology and field of education, simulation applications and simulation tools provides increase in technical and non-technical skills in nursing education (Goris et al., 2014). Described as a teaching method used to increase real life experiences, simulation has gained a ground in nursing education at the level of bachelor's degree within the frame of skill development (Brown, 2015). The opportunity of applying theoretical information in practice and decreasing the difficulties that students face in clinical environments can be found by means of simulation trainings (Terzioglu et al., 2012).

The skills required to be gained in mental health and diseases nursing education vary. The clinical environment can be animated close to real life with the simulations used in this field. It provides gaining consistent, comparable, reproachable, and discussable experiences with active learning (Durham & Aldem, 2007). Biases, stigmatization, anxiety and fear etc. cases may prevent gaining these experiences by means of real patients. It was determined in a study carried out in Turkey that 73,9% of nursing students at the level of bachelor degree have not made contact with a patient with a mental disorder before taking the course of mental health and diseases and 34,4% of the students entering into psychiatry clinical practices are scared during practices and 41,7% are demoralized (Ozbas & Buzlu, 2011). Besides these, students cannot encounter all cases during practices as the circulation of patients is high, the staff is insufficient, clinical practice hours are inadequate or they may have some difficulties in learning because of the policies of health institutions limiting the studies of student nurses. For these reasons, use of simulation in nursing education has gained importance (Brown, 2008; Brown, 2015; Guise et al., 2012).

Literature Information

The design and method of simulation should be elaborated for ensuring successful outcomes of simulation applications having great importance in nursing education (Hammer et al., 2014). "The Framework of Design, Application and Assessment of Simulation used in Nursing Education" was formed by Jeffries in 2005. There are five significant variables in the framework that are related to one another. These variables cover educational applications, teacher, student, design features of simulation and conclusions (Grom et al., 2013; Jeffries, 2005). Use of simulation method used in nursing education within the framework will provide the increase in the efficacy of simulation.

Students manage the simulation by themselves by playing the roles of patient, patient's relative, healthcare practitioners. Trainers facilitate,

formalize, and support the application of simulation with questions and answers and their observations. The phase of resolution, which is defined as debriefing, enables the correction and improvement of targets, theories and errors within the guidance of the faculty (Fay Hilier, 2012). It is required that the skill steps included in simulation assessment guide should be clear, understandable, valid and reliable both for educators and students (Sarıkoç et al., 2016).

Psychiatric nursing students have the opportunity of learning psychiatric interventions and crisis management in a terminal environment being tranquil and positive during patient/relative care by using simulation applications. Simulation applications benefitted in the education of psychiatric nursing students improve, therapeutic education skills, critical thinking, decision making, ceasing the interventions applied, interactive learning, problem solving, using themselves in therapeutic manner, crisis management, communication skills, self-sufficiency, self-confidence, interviews, cultural sufficiency and thinking/decision making capabilities in the clinic of students (Brown, 2015; Crider & Niesh, 2011; Guise et al., 2012; Haracz et al., 2015; Kameg et al., 2009; Szpak, 2013). Simulation applications increase the students' levels of perception of clinic and facilitate the understanding of psychiatry patient and the surrounding (Hermanns et al., 2011; Qudshoorn & Sinclair, 2015).

Simulation applications conducted by preparing different scenarios provide students with the chance of being assessed together with their peers without being judged by bringing a concrete and emotional experience (Ellis et al., 2015). In the phase of debriefing, students become aware of their negative attitudes and express their emotions and opinions and can achieve self-management (Hermanns et al., 2011; Guise et al., 2012; Murray, 2014). Educators cannot make adequate observations to assess students and improve their knowledge and skills in psychiatry clinics where students interact with patients because of the reasons such as confidentiality and environmental problems (Qudshoorn & Sinclair, 2015). However, educators can discuss the care delivered to the patient by students in psychiatric patient care thanks to simulation applications and can explain their expectations. In addition to this, educators can observe students individually and by this means, they can detect the powerful and weak aspects of students and thereby provide empowering students (Ellis et al., 2015). When simulation applications are used in accordance with learning targets, the target is achieved in fast and more reliable manner (Guise et al., 2012).

Simulations training also have some disadvantages besides the advantages and for that reason, these needs to be used meticulously. The cases shall be selected compliant with the target in simulation training; trainings shall be conducted at proper times; the number of students shall be ideal;

the equipment used shall be sufficient; the propriety and scope of the field shall be evaluated and the phase of resolution shall be managed well. Use of standardized patient or manikins and use of high precision manikins increase the cost and adapting these manikins to different scenarios; simulator's responding to the applications performed and the accompanying equipment also result in the increase in cost (Brown, 2008).

Simulations are conducted using quite different techniques in mental health nursing education as well as health education. Simulations with standardized patients, high precision human simulation, virtual patients (realism) and audio simulations are among the simulations most commonly used in mental health nursing education (Brown, 2015; Goris et al., 2014; Guinnes, 2011; Guise et al., 2012; Sendir & Dogan, 2015). This study targets examining the studies analyzing the effects of simulation method conducted in psychiatric nursing education through standard patient on students.

METHOD

The systematic review was conducted according to the standards of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Figure 1) (Moher et al., 2009). PROSPERO does not currently accept registrations for scoping reviews, literature reviews or mapping reviews. Therefore the registration number could not be obtained.

Study identification

Overall, we examined 1112 papers (Figure 1). Because of the studies searched in PsychINFO (277 studies), PsychARTICLES (49 studies), PubMED (137 studies), CINAHL (186 studies), OVID (147 studies), Clinical Key (267 studies) and The COCHRANE LIBRARY (49 studies) database in 2005-2021, 85 articles, which were conducted on nursing students, in the scope of the course of mental health and disease nursing were taken into assessment. Searches in databases were made by creating appropriate combinations with MeSH terms. These combinations; Psychiatric nursing and simulation consisting of standard patients in psychiatric nursing education, standard patient in nursing education, and simulation in nursing education. 65 studies that do not use standard patients, which is one of the simulation methods in simulation trainings, and whole text of which could not be accessed were not included in the research. 20 studies using simulation method with standard patient were included in the research and analyzed (Figure 1).

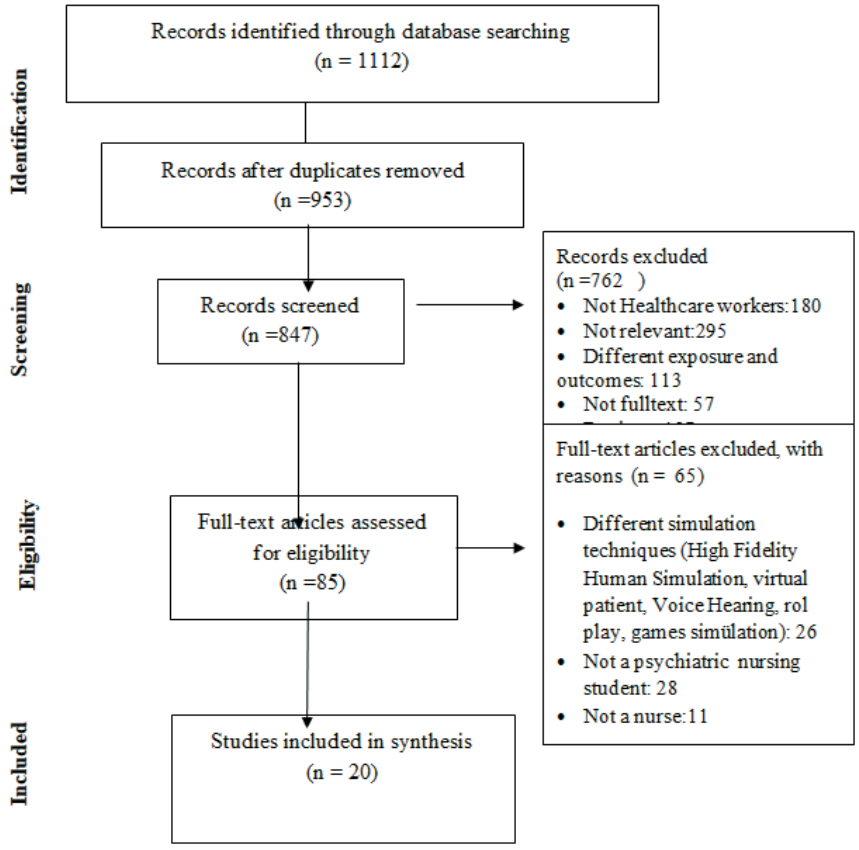

Figure 1. Study flow diagram.

Study Eligibility

The abstract screening process followed the predetermined inclusion criteria: involving standardized patient, peer-reviewed, research study of any kind (all study designs included), experimental studies, written in the English language, were delivered to nursing students, and from 2005 to 2021. Additionally, we excluded articles that did not provide a description of the sample, articles that were not primary research studies, dissertations, simulation were not used as an intervention, or had insufficient details about the simulation methods. Articles were screened independently by two reviewers. All discrepancies between authors resolved by the third reviewer.

Findings and Discussion

When the design of the studies included in the study is considered, it is seen that 6 qualitative method, 11 quantitative methods, and 3 qualitative

and quantitative method used together (Table 1). The studies included in the study were analyzed under the themes of scale themes used with the purpose of assessing the effect of simulation and the scenarios used, and the effect of use of simulation on psychiatric nursing students.

Standard Patient Simulation - Standardized Patients (Sps)

Standard patient simulation is an education method which is facilitating learning and student-centered and in which theoretical knowledge and clinical knowledge and experience are gathered. Individuals trained for standardized patients describe a medical case or a case of disease in a true and consistent manner with the purpose of providing students the opportunity of applying clinical skills and observing their applications. Standard patients are selected by taking age, gender and body size into consideration to comply with the scenario planned to be animated and the people to be animated are required to have skills closest to real. It is necessary that standard patient shall show the same performance in all animations (Adamo, 2003). Anxiety is seen very often in students going to mental health clinical practices. The standardized patient method decreases the anxiety of students encountering patients with mental diseases in real life and increases self-confidence. In studies conducted with the students having experienced standardized patient simulations, which is one of the mental health simulations, it was found out that this simulation method develops students' experiences of contacting patients, skills of assessing the complaints of patients, therapeutic communication skills, and sense of security, and decreases the level of anxiety (Brown, 2015; Choi, 2013; Davis et al., 2013).

Effects of Use of Simulation on Students

The researches included in the study were analyzed in terms of evaluating the effect of use of simulation on students (Table 2). The studies expressed the importance of use of simulation in psychiatric nursing education and notified that simulation has positive effects. It was determined in a study that the experimental group working with standard patient has less negative attitudes toward schizophrenic patient compared to the control group and students in the experimental group feel less frightened and threatened. Additionally, it was stated that the students working with standard patient intend to show any behavior to interact with the patients when they come across in clinic. In the same study, it was specified that the students having higher levels of fear interact with patients less often in clinical practice (Sideras et al., 2015). It is observed that simulation applications having been determined to decrease the fear and to increase the interaction with patient shall take place among education methods to

provide ideal patient-nurse interaction, being one of the main objectives of psychiatric nursing.

In 5 of the 20 (25%) studies reviewed, therapeutic and nontherapeutic communication skills were evaluated and it was notified in all studies that simulation trainings have positive effects on communication skills in statistically significant manner (Martin & Chanda, 2016; Sedghi et al., 2014; Webster, 2013; Webster, 2014). Use of simulation in teaching psychiatric nursing and therapeutic communication techniques is a new pedagogy having a multidisciplinary potential. It is suggested that simulation, aiming at developing communication techniques must be applied prior to commence of clinical practices in psychiatric nursing education and assessments must be made by means of standard tools. Communication evaluation scales used and checklists developed are used for that (Hammer et al., 2014). Awareness is created in students with simulation applications performed in different environments concerning patient and communication techniques and in this way, establishing therapeutic communication with patients gets easy (Karlsen et al., 2017). Increasing simulation applications performed with standard patients used for enhancing communication skills of nurses and facilitating their interaction with patients is proposed with the methodological studies conducted meticulously (Karlsen et al., 2017; Maclean et al., 2017).

In a researches included in the study, self-efficacy and attitude (Alfes, 2015) were evaluated and bias, self-efficacy and ability to feel empathy (Choi et al., 2016) Knowledge and perceived competency of the nursing care of a patient diagnosed with schizophrenia (Speeney et al., 2018) were assessed in another research. Alfes (2015) studied with standard patient animating depression scenario in the study and divided students in four groups. Self-efficacy of students were assessed and it was determined that the level of self-efficacy of all groups increased in time and there was not any difference between groups ($F = 46.924$, $df = 1.298$, $p \geq 0.05$). Choi et al. (2016) divided the students in two groups and tasked both groups with two scenarios and evaluated their level of empathy, self-efficacy and bias about the mental disease. In the end, it was notified that there was improvement in the level of empathy and self-efficacy after clinical practice, but there was not any change about bias. In standard patient simulation method, students can both play the role of patient and nurse (Sendir, 2013). Thanks to this technique in the simulation, students can feel the psychotic symptoms of patients by experiencing it as they have the role of patients and can be aware of what patients live and think besides identifying the symptoms. By this means, empathy skills of students are improved (Choi, 2013). Besides this, permanence of student's skills and knowledge is increased with simulation applications used in psychiatric nursing education and their level of self-efficacy is increased. Students with high level of

self-efficacy show better performance in terms of attitudes towards patients (Kunst et al., 2016).

Communication and anxiety levels were evaluated in two of the researches included in the study and level of anxiety was assessed in one another (Kameg et al., 2014; Ok et al. 2020; Webster 2014;). Kameg et al. (2014) used STAI and VAS anxiety rating scale and simulation evaluation survey and indicated that there is a relationship between levels of instant and continuous anxiety. Robinson Smith et al. (2009) They evaluated students in the study with student satisfaction survey and self-confidence survey and in conclusion, students specified that standard patients are quite close to real patients, feedbacks contributed them much and decreased their level of anxiety and anger that they had before simulation. It was also detected in different studies that the level of anxiety of students decreases with the simulation methods and accordingly, their self-confidences increase (Dearmon et al., 2013).

In a study, the students' motivation and their level of perception were evaluated. (Sarikoç et al., 2017). It was concluded that standard patient practices have some positive effects on their motivation and level of perception. In another study, the subjects in which the students have lack of information were determined. Student satisfaction was seen to be high after the research (Witt et al., 2018). As they are among the active learning methods, simulation practices involve the students in the learning process as well. This situation can boost student satisfaction.

Qualitative design was used in 6 of the studies included in the study (Table 2). Focus discussion method was used in one of these qualitative design studies and efficiency of simulation was assessed (Lang & Hahn, 2013). Communication skills were assessed in the other one (Webster, 2013) and the issues of importance of debriefing with descriptive analysis, gaining insight and increase in confidence were expressed in the last one (Schwindt et al., 2015). Students are encouraged to express their emotions and thoughts during debriefing and the comments belong to the observations of their peers and professionals during the planned sessions ensure obtaining positive results (Schwindt & McNelis, 2015). In addition to this, students stated that they realize their powerful and weak aspects (Webster, 2013), their beliefs, values, decisions related to mental disease and non-therapeutic techniques performed (Lang & Hahn, 2013; Webster, 2013); the gap between theory and practice is filled with simulation (Alexander et al., 2018; Jacobs & Venter, 2017); humiliating attitudes towards psychiatric patients reduce (Alexander et al., 2018); they gain positive learning experiences in a real and safe atmosphere (Jacobs & Venter, 2017); they have less assumptions and worries about psychiatry nursing; they gain information about daily lives of the patients (Knutson de Presno et al., 2021);

and hearing the comments whether the specific conducts and actions taken are beneficial or not directly is quite advantageous (Schwindt & McNelis, 2015).

It is expressed that forming discussion areas, watching the videos again, giving feedback and assessments of teachers and peers in simulations containing similar or different clinical experiences to increase knowledge and skills of students contributes to the development of their insight (Schwindt & McNelis, 2015; Webster, 2013). Students' having the opportunity of eliminating their shortcomings and developing knowledge and skills with these studies provides an increase in their self-confidence (Schwindt & McNelis, 2015). It is specified by students in the feedback given that it is an efficient method to understand patients with psychological disorder, to make contact with patients and to improve existing communication (Oudshoorn & Sinclair, 2015). Doolen (2014) evaluated the demands of students in the study and students expressed that standard patient practices must continue in each school year and be integrated in school years by increasing the number of cases. Besides being a new model to improve students in classroom and clinical environment with the use of standard patient in psychiatric nursing education, it is also an efficient teaching method improving patient-nurse relationship (Lang & Hahn, 2013).

Scenarios Used in Simulation

Although the simulation method with standardized patient is subjected to various criticisms due to wide diversity, it is the most common method in mental health nursing students. Standardized patient scenarios are increased to be used in this field by using DVD and video combinations to create visual and aural short stories (Guise et al., 2012).

Scenarios are created in simulation training studies conducted with standard patient and the trainings related to these scenarios are presented to the ones who will animate standard patients. Trainers can turn the cases into a real simulation scenario by benefitting from their experiences. It is important that the symptoms identifying the disease must be clear, understandable and explicit in scenarios (Sarıkoc et al., 2016). The scenarios that the researches included in the study used are given in Table 1. 34 scenarios were used in total as follows: depression was used in 10 studies (Alfes, 2015; Choi et al., 2016; Jacobs & Venter, 2017; Kameg et al., 2014; Lang & Hahn, 2013; Martin & Chanda, 2016; Oudshoorn & Sinclair, 2015; Robinson Smith et al., 2009; Sarikoç et al., 2017; Schwindt & McNelis, 2015), chronic schizophrenia was used in 10 studies (Alexander et al., 2018; Choi et al., 2016; Doolen et al., 2014; Lang & Hahn, 2013; Martin & Chanda, 2016; Ok et al., 2020; Oudshoorn & Sinclair, 2015; Sideras et al., 2015; Speeney et al., 2018; Webster, 2013), anxiety disorder was used in 4 stud-

ies (Choi et al., 2016; Doolen et al., 2014; Kameg et al., 2014; Oudshoorn & Sinclair, 2015;), bipolar disorder was used in 4 studies (Doolen et al., 2014; Knutson de Presno et al., 2021; Martin & Chanda, 2016; Webster, 2013), psychotic patients with active suicide ideations were used in 1 study (Schwindt & McNelis, 2015), patient with drug use disorder was used in 1 study (Oudshoorn & Sinclair, 2015), borderline personality disorder was used in 1 study (Choi et al., 2016), eating disorder with suicide attempt was used in 1 study (Lang & Hahn, 2013), OCD was used in 1 study (Lang & Hahn, 2013), no scenario was stated in 1 study but the students were stated to carry out evaluations of mental health assessment, therapeutic communication skills, cognitive processes, assessment, of mood/affect (Witt et al., 2018). The periods having both manic and depressive attacks of a patient with bipolar disorder were separately studied in 1 study. As it is seen in Table 1, scenarios used in studies contain more than one case and it shows that these are applicable in several cases by offering chance for comparative studies.

CONCLUSION

In conclusion, use of appropriate simulation methods decreases various negative effects to develop before experiences clinical experience particularly and contributes to both students and teachers positively as learning theories in the education of the students to work with mental disorders are also supported as specified in the literature. Prepared by using different scenarios, simulation applications can be used in the treatment of several cases. It is suggested that experimental studies should be conducted on the use of simulation in psychiatric nursing education and awareness about the subject should be raised.

Table 1 *Design of Studies and Scenarios Used*

	Author and year	Type of study	n	Simulation duration	Scenario
1.	Sideras et al (2015)	Quantitative research	145	60 min	• Schizophrenia with auditory hallucinations
2.	Sedghi et al (2014)	Quantitative+ Qualitative research	30	15-30 min	• Psychotic patient
3.	Webster (2014)	Qualitative research	Unspecified	15-20 min	• Paranoid schizophrenia • Bipolar disorder (manic and depression epizod)
4.	Webster (2013)	Quantitative research	89	15-20 min	• Paranoid schizophrenia, obsessive compulsive disorder, bipolar disorder, suicidal thoughts, borderline personality disorders, dementia
5.	Martin and Chanda (2016)	Quantitative research	28	5 (scenario)+ 40 min(debrifing)	• Depression • Bipolar Disorder • Schizophrenia
6.	Alfes (2015)	Quantitative research	77	10 min+ 15 min	• Depression
7.	Choi et al (2016)	Quantitative research	22+22		• Schizophrenia • Generalized anxiety disorder • Borderline personality disorder • Major depressive disorder
8.	Kameg et al (2014)	Quantitative research	69	5 min+ 30 min debrifing	• Anxiety and depression
9.	Robinson Smith et al (2009)	Quantitative research	112	15 min	• Depression
10.	Schwindt and Angela McNelis (2015)	Qualitative research	15	30 min	• Major depression • Patient with active suicidal thoughts and psychotic symptoms
11.	Oudshoorn and Sinclair (2015)	Quantitative research	56	30 min (scenario)+ 45 min (debrifing)	• Anxiety disorder • Schizophrenia • Substance use • Depression
12.	Lang and Hahn (2013)	Qualitative research	Unspecified	15 min	• Suicide-induced eating disorder • Depression • Schizophrenia • Obsessive Compulsive Disorder
13.	Doolen et al (2014)	Quantitative+ Qualitative research	94	20 min	• Schizophrenia • Anxiety • Bipolar disorder
14.	Speeney et al (2018)	Quantitative research	52	Unspecified	• Schizophrenia
15.	Ok et al (2020)	Quantitative research	85	10-12 (scenario)+ 30-35 min(debrifing)	• Schizophrenia
16.	Alexander et al (2018)	Qualitative research	13	Unspecified	• Schizophrenia. The SP presented as delusional and paranoid
17.	Jaobs and Venter (2017)	Qualitative research	33	Unspecified	• Depression
18.	Sarikoç et al (2017)	Quantitative research	86	Unspecified	• Depression (‘a patient with depression having suicidal ideation’ and ‘a patient with hallucinations’)

19.	Witt et al (2018)	Quantitative+ Qualitative research	32	120 min	• Mental health assessment, therapeutic communication skills, cognitive processes, assessment, of mood/affect.
20.	Knutson de Presno et al (2021)	Qualitative research	24	30 (scenario)+ 20-30 min(debrifing)	• Bipolar disorder (manic and depression epizod)

Table 2 *Results of use of Simulation*

	Author and Year	**Scale used**	**Findings and Results**
1.	Sideras et al (2015)	Fear and Behavioral Intentions (FABI), Attribution Questionnaire (AQ-9)	• Differences in negative attitudes against schizophrenic patient were determined at significant levels in experimental group compared to control group and experimental group specified that they experience less fear and feel less threat and significant differences were revealed. • It was notified that the students having high level of fear interacted with patients less in clinical practices.
2.	Sedghi et al (2014)	Communication skills survey	• Significant differences were detected between therapeutic communication skills before and after the simulation training and n 75% (67-100) of participants after simulation training ($t = -22.530$, $p < 0.001$). • There was not any difference in increase in verbal and non-verbal communication skills after simulation and simulation affected both at the same rate.
3. eac	Webster 2013	Communication skills survey	• They expressed during debriefing that watching themselves contributes them in terms of realizing wrongful behaviors of them. • In debriefing, students were asked to explain how they reveal the values of patient and students expressed that they realized the importance of listening to patients and receiving feedbacks without judging.
4.	Webster (2014)	Communication evaluation survey, anxiety level assessment	• After the first standard patient simulation, it was observed that students' communication skills were improved and there was difference between both applications. Besides this, students specified that their anxiety decreased ad their self-confidence increased during the application of second standard patient simulation.
5.	Martin and Chanda (2016)	Communication skills scale	• The same scale was used before and after the simulation. They were graded according to communication techniques used when encountered with standard patient and difference after simulation application was detected in a manner that it is significant statistically between pretest and posttest.
6.	Alfes (2015)	Attitudes to Mental Illness Questionnaire (AMIQ)	• Self-efficacies of students were measured in mental health clinic and expressed that they feel themselves having more self-efficacy. • Students were divided into four groups and the level of self-sufficiency of all groups was increased in time while there was not any difference detected among groups ($F = 46.924$, $df = 1.298$, $p \geq 0.05$). • There was not any difference between groups in attitude scale and it was specified that there was improvement in all groups in time.

7.	Choi et al (2016)	Questions About the Mental Health Clinical Practicum Empathy Construct Rating Scale, Social Stigma Scale of Mental Illness Patients, Self-Efficacy scale	• While there was improvement concerning empathy and self-efficacy after clinic practice, there was not any change related to bias, and the expectations and assessments of the students were reported.
8.	Kameg et al (2014)	Anxiety visual analog scale and the State-Trait Anxiety Inventory (STAI)	• Anxiety levels were measured before and after simulation and in conclusion, high level of relationship was found among instant and permanent anxiety. • Positive feedbacks were received from the students attending simulation application.
9.	Robinson Smith et al (2009)	Student satisfaction questionnaire and self-confidence questionnaire	• Approximately about 45% of students stated that standard patient provided real application, 20% specified that feedbacks are significant and 23% said the status of anxiety and nervousness existed before simulation decreased. • Students notified that their self-confidence is better while assessing the patient and they used positive communication techniques.
10.	Schwindt R and Angela McNelis (2015)	Questionnaire forms	• Descriptive analysis method was used. Students revealed three themes by expressing the significance of simulation training. • Themes: importance of feedback, gaining insight and increasing confidence.
11.	Oudshoorn and Sinclair (2015)	Reflective practice review (RPR)	• In the study, the objective of which is teaching mental health concepts, the subjects of assessing the risk of suicide, evaluating mental health, supporting patients during crisis, preparation for discharge and supporting the patients experiencing mental change were studied. • It was specified by students that the study carried out was quite efficient for helping improvement of their relationships with patients and understanding the course of patients with mental disorder.
12.	Lang and Hahn (2013)	The efficacy of simulation of focus group discussion was assessed.	• Used model was notified that this model was a new model to improve students in classroom and clinical environment, use of standard patient develops patient and nurse relationship as an influential learning and teaching method and this model can be used in courses, simulation applications and conferences by adopting it to nursing bachelor's degree.
13.	Doolen (2014)	Simulation evaluation form and feedback report	• It was concluded that students demand continuation of standard patient applications and increasing the number of cases and integrating it into each school year.
14.	Speeney et al (2018)	knowledge and perceived competency of the nursing care of a patient diagnosed with schizophrenia	• According to the results of the study, an increase was observed in the perceived competence and knowledge levels of the students.
15.	Ok et al (2020)	Communicational skills inventory (CSI) State-Trait Anxiety Inventory (STAI)	• According to the results of the study, it was observed that the anxiety levels of the students in the experimental group decreased and their communication skills improved.

16.	Alexander et al (2018)	Semistructured focus group exploring their perceptions of mentally ill persons and their experiences of simulation	• According to dec results of the qualitative research conducted; It was stated by the students that the gap between standard patient practices and theoretical knowledge and clinical practice has been closed. • It turned out that simulation has a role in decreasing of the students' humiliating attitudes towards psychiatric patients.
17.	Jacobs and Venter (2017)	Signed informed consent forms	As a result of their thematic analysis, they have revealed the following themes. • Positive learning experience • Realistic and safe environment • Integrating theory with practice • Professionalism • Confidence • Processing skills in communication
18.	Sarikoç et al 2017	Student Information Form", "Motivation Scale" and "Perceived Learning Scale" (pre-test).	• Using standardized patients in psychiatric nursing education had positive impact on motivation and perceived learning of the students.
19.	Witt et al (2018)	Satisfaction Surveys, and behavioral checklists.	• It was stated that the students most commonly had lack of information about evaluating the patients with the conditions of bipolar disorder, symptoms of lithium toxicity, stress disorders after trauma and dementia phases. They expressed that they were happy working with standardized patients
20.	Knutson de Presno et al (2021)	Semistructured focus group	As a result of their thematic analysis, they have revealed the following themes. • Preview into everyday life in a psychiatric ward, • Adjusting assumptions and apprehensions regarding mental health nursing and, • Mutual respect during the nurse-patient meeting.

LIMITATIONS

Only studies written in English are included in the review, which may led to a publication bias. The study focused on a single simulation type. Different types of simulations are excluded. This limited the number of studies.

RELEVANCE TO CLINICAL PRACTICE

This study provides information about the use of standard patient simulation methods in psychiatric nursing education. It gives information about the designs of the studies conducted in this field and the scenarios used. It presents scenarios that can be used in standard patient simulation and their effects on students. Evaluates standard patient-related educational practices, informs educators about standard patient use and gives ideas for new research.

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CHAPTER 10

SUSTAINABLE NUTRITION AND ENVIRONMENTAL EFFECTS

Nurgül ARSLAN[1]

1 Doktor Öğretim Üyesi, Dıcle Unıversıty Atatürk Faculty Of Health Scıences, Department Of Nutrıtıon And Dıetetıcs ORCID: 0000-0002-7618-0859

SUSTAINABLE NUTRITION

The idea of "sustainable nutrition," which is derived from sustainable agriculture, aims to reduce waste of natural resources and to produce enough food for seasonal and natural consumption. Sustainability as an idea is nothing new (Qaim, 2017). The Brundtland Commission, formerly known as the World Commission on Environment and Development, coined the terms "sustainability" and "sustainable development" in 1983. Sustainable development is defined by the Brundtland Commission as "meeting the needs of the current generation without compromising the ability of future generations to meet their own needs" (Smetana, Bornkessel, & Heinz, 2019).

In the late 1990s, the Conference of the Parties (COP), which oversees the Convention on Biological Diversity (CBD), acknowledged the significance of biodiversity for food safety. The need for sustainable biodiversity use in the fight against malnutrition and hunger, as well as the link between biodiversity and nutrition and nutrients, were both formally acknowledged by the COP in 2004. In 2010 this initiative was combined with sustainable diets after being introduced two years earlier and intersecting with biodiversity for nutrition and nutrients (Burlingame, 2014). Over the course of the past two decades, the idea of sustainable nutrition has progressed further. The Food and Agriculture Organization (FAO) worked hard in 2010 to develop the definition of'sustainable nutrition,' and while it is not universally agreed upon, this is what they came up with: Diets that are sustainable have a low impact on the environment and contribute to the food and nutrition security as well as to living a healthy life for both the current generation and future generations (Durazzo, 2019). Diets that are sustainable protect and show respect for biodiversity and ecosystems; they are also culturally acceptable, easily accessible, economically viable, and cost-effective; nutritionally adequate, safe, and healthy; and they are the diets that make the most efficient use of both natural and human resources (Lairon, 2012). This definition demonstrates that human health and ecosystems are not two separate entities that can be considered independent of one another. For instance, one's health may be contingent upon the affordability of food as well as one's access to food of sufficient quality; alternatively, the quality of food may be contingent upon the land and soil (that is, the environment) in which it is produced (Qaim, 2017). The study of nutrition concentrates primarily on individual nutrients, nutrient deficiencies, food groups, and the effects these have on one's health. Recent years have seen an increased emphasis placed on diets and the effects those diets have on human health, the environment, and food systems (Tobi et al., 2019). A great number of economic, social, cultural, and environmental factors can have an impact on food systems, which also have a high degree of complexity. The relati-

onship between shifts in the environment, shifts in food systems, and shifts in food security is a two-way street. The processes and outputs of food systems are one of the most important factors contributing to environmental changes. Changes in the environment can have an effect on the functioning of food systems in terms of the safety, productivity, and quality of the foods that are produced, and one of the most important factors contributing to environmental changes is the processes and outputs of food systems (Grosso, Mateo, Rangelov, Buzeti, & Birt, 2020). In every region of the world, specific plans for food production and consumption are required in order to guarantee food safety without putting additional strain on the environment (Alsaffar, 2016). Nutrition and food security are important policy issues in every country, and the current global malnutrition crisis is a concern in both developed countries and developing countries alike. It is estimated that thirty percent of the world's total greenhouse gas emissions are caused by the current global food system. The Foresight Project; It is estimated that by the year 2100, the global population will reach at least 9 billion people. The contribution of food and agriculture to environmental degradation and climate change is expected to increase as a result of the growing demand for the transportation and storage consumption of foods that contain high levels of nutritional components such as meat and milk in developing economies (Fanzo, Bellows, Spiker, Thorne-Lyman, & Bloem, 2021).

The idea of sustainable nutrition has been neglected, along with the sustainability of ecosystems, as agricultural production has increased as part of the process of globalization of food. This has led to a lack of attention being paid to the concept of sustainable nutrition. However, as of late, there has been a rise in interest in various diets, which has led to this problem being brought to the forefront of international scientific associations and institutions in a number of European nations (Shepon et al., 2021). Along with the difficulty of defining what it means to be sustainable, there is also mounting evidence that the diets that are practiced today are not sustainable. Although the entire nutrient system is included in the definition of sustainable nutrition, it is imperative that it be understood that food production systems, in addition to nutrient and nutritional requirements, are interdependent on one another. A sustainable diet entails cutting back on overconsumption and making the shift toward a healthy diet that has a smaller negative impact on the environment. It also means lowering the amount of waste and nutrient runoff produced by food systems (Reyes et al., 2021). The transition to a diet that is healthy and sustainable should have as its end goal the preservation of the health of both people and the ecosystem. These kinds of fundamental shifts might call for fundamental adjustments to the food system. It is true that one of the goals is to reduce

the amount of greenhouse gases that are produced while food is being produced; however, this alone will not be sufficient for the reduction in greenhouse gases; consequently, dietary intakes will need to be altered in order to achieve the targets that have been established. It is necessary to define dietary changes in order to achieve a healthy diet that has a reduced impact on the environment. The most difficult challenge is figuring out how to convince people to make these adjustments and alter their existing patterns of eating (Grosso et al., 2020; Lairon, 2012; Qaim, 2017). Food consumption patterns are not only a reflection of a person's dietary requirements, but also of their cultural and ethnic preferences, as well as their preferences regarding taste, smell, and texture. For this reason, environmentally responsible patterns of food consumption should take into account not only pollution and waste, but also cultural and intangible qualities. Historically, eating habits were determined by the availability of locally produced foods; however, as time passed, unusual fruits, vegetables, and spices gradually became more prevalent (El-Ramady, Olle, Eichler-Löbermann, & Schnug, 2020). Food consumption patterns of the future will be a reflection of general lifestyles, income levels, and values; however, the impact that these patterns have on the environment should not be disregarded (Guillaumie, Boiral, Baghdadli, & Mercille, 2020).

EFFECTS OF NUTRITION AND NUTRITIONAL SYSTEMS ON THE ENVIRONMENT

Nutrition and greenhouse gas

Emissions of greenhouse gases into the earth's atmosphere, which originate from human activities and are active in terms of radiation, change the structure of the atmosphere and have effects on the balance of radiation in the atmosphere, and as a result, on the climate of the entire planet. Emissions of carbon dioxide, methane, and nitrous oxide are the primary contributors to the greenhouse effect caused by human activity (Jessica L Johnston, Jessica C Fanzo, & Bruce Cogill, 2014; Qaim, 2017). The United Nations Framework Convention on Climate Change called for "keeping the greenhouse gas concentration in the atmosphere at a certain level will prevent dangerous anthropogenic interference with the climate system" in 1992. The developed countries were the first to do this in 1997 in Kyoto. once this was accepted, the convention went into effect (Gustafson et al., 2016).

It is estimated that climate change will have major effects on human health, and global warming will be one of those consequences. It is hypothesized that agriculture and, consequently, changes in land use are responsible for approximately thirty percent of the world's total greenhouse gas

emissions (Dornhoff, Hörnschemeyer, & Fiebelkorn, 2020). Agriculture is one of the factors that contributes to the degradation of land and the emission of greenhouse gases caused by humans. Carbon 25%, 50% of methane, and more than 75 percent of nitrous oxide are emitted as a result of human activity (Pawlak & Kołodziejczak, 2020). When combined with the effect of land use, the impact of food systems in the UK is estimated to reach as high as 30% of total greenhouse gas emissions. In the UK, food systems are responsible for approximately 19% of greenhouse gas emissions. When looking at Europe's total greenhouse gas emissions, agricultural production and food consumption account for approximately 20-30% of those emissions, and the food system is Europe's largest industrial sector (Aceves-Martins et al., 2022). Estimates of greenhouse gas emissions take into account emissions produced during all stages of the life cycle of a product, including production, transformation, distribution, use, and disposal of the product. An additional method is the input-output analysis, which is utilized to estimate the effects that products and services have on the environment (J. Macdiarmid & Whybrow, 2019; Soergel et al., 2021).

As a consequence of this, an estimation of the typical environmental impact of a certain product group is made. In today's world, there are techniques that can take the life cycle analysis and add an input-output analysis to it (Monteiro et al., 2018). The production of food and the ways in which it is consumed place a strain on the environment. Processing, storing, transportation, distribution, and waste are all aspects of the food production life cycle that have an effect on the surrounding environment. The extraction and utilization of natural resources, the release of greenhouse gases into the atmosphere, pollution, irresponsible use of natural resources, the consumption of energy, and the generation of waste are all components of this effect (Gil et al., 2019). When making decisions with regard to a product's potential impact on the environment, it can be helpful to examine the product's entire life cycle. For instance, decreasing the amount of refrigeration that a product receives can cut down on the emissions produced during storage but will lead to increased nutrient waste. One of the environmental impacts of nutrition is the production of greenhouse gases, but it also has an effect on the way water is used, the diversity of plant and animal life, and the shape of the land (Burlingame & Dernini, 2010; Pawlak & Kołodziejczak, 2020).

Greenhouse gas effects of foods

Dietary guidelines that combine maximum and minimum intake recommendations form the foundation of healthy diets. Over the past 50 years, dietary habits have drastically changed across the globe. People now eat foods that are bad for their health and the environment. Meat, milk,

oil, salt, and processed food consumption all rise in tandem with rising national and individual incomes. The high consumption of meat and dairy products, as well as the rise in their production, especially in high-income countries, have a negative impact on climate change (Comerford, Miller, Reinhardt Kapsak, & Brown, 2021). Some nations have created sustainable diets with minimal negative effects on the environment and manageable greenhouse gas emissions. For instance, new diets that limit meat consumption and favor sustainably farmed fish have been developed in the Netherlands and Sweden (Fresán & Sabaté, 2019). These diets typically restrict animal foods because of the significant environmental impact of animal products. In Western culinary tradition, meat plays a significant role in meals. Vegetable protein has remained stable over the past ten years, while meat consumption has steadily increased (Lutz, 2021). In the coming years, there will likely be a significant increase in the demand for animal products, and between 1999 and 2050, the world's meat production is expected to more than double. In the last five years, the consumption of foods derived from animals has increased from 15.4% to 17.7%. A closer look reveals that the majority of this growth is attributable to emerging economies and developing nations, with developed nations' share remaining nearly constant. When it comes to biodiversity loss, greenhouse gas emissions, and other environmental burdens, the use of water and land for the production of animal foods is one of the most damaging processes for the environment. Fruit and vegetable production is less responsible for the rise in greenhouse gas emissions, water use, and energy consumption than the production of foods derived from animals, such as meat, fish, and dairy products (Razzaq, Tang, & Qing, 2021). Experts in environmental science claim that a diet with fewer animal products results in lower greenhouse gas emissions. The eating of animal foods is complicated, though. Meat products are a good source of nutrients because they contain high-quality protein and important micronutrients, but red meat, particularly processed meats, is also linked to an increased risk of developing certain chronic diseases (Agnusdei & Coluccia, 2022). Ecosystems may be under stress, and it may also have a negative impact on climate change. Livestock is thought to be responsible for 14.5% of greenhouse gas emissions that contribute to climate change. While increasing productivity, advancing technology, and reducing food waste are the cornerstones of strategies to combat climate change, it is becoming more and more apparent that dietary changes are also necessary to meet greenhouse gas emission targets. A diet low in meat products may therefore be both healthier and have less of an impact on the environment, indicating that a sustainable diet may be compatible with public health (Comerford et al., 2021; Grosso et al., 2020). The suggested diets may need to have their nutritional content examined, though. because animal foods are a great source of nutrients. It may be challenging

to reduce the consumption of animal products at the population level in nations where nutrient deficiencies are common. White meat consumption was found to be protective in two sizable studies, whereas consumption of red and processed meat was linked to diseases. The nutritional value of chickens can vary depending on the production methods used. For instance, it was emphasized that factory-farmed chicken had meat that was roughly one-third more fatty than chicken that had been raised outdoors. Health issues associated with intensive production may also include the overuse of antibiotics and food safety (Ong et al., 2021). Consuming fish is associated with a lower risk of cardiovascular disease. One serving of oily fish may be enough to lower the risk of cardiovascular disease, according to some scientific opinions. But most artificial ecosystems claim that fish come from nature and that fish stocks are in danger. Loss of biodiversity and reduced catches have been observed in the last 20 years as hunting has shifted from carnivorous fish to less abundant herbivorous fish. According to estimates, 30% of fish stocks are overfished or depleted while 50% of fish stocks are being used up completely. The type of fish eaten, where it comes from, and the methods of fishing all have an impact on the environment. Compared to wild fish, farmed fish may not always be ecologically sustainable (Aceves-Martins et al., 2022; J. I. Macdiarmid, 2022).

A diet rich in plant-based foods is crucial for overall health. It includes nutrients like carbohydrates, dietary fiber, and vitamins that may be good for your health. Antioxidants and phytochemicals are also present. More plant-based diets have been linked to lowered disease risks. Dietary recommendations urge an increase in plant-based foods because of these factors. Plant-based diets produce fewer greenhouse gas emissions than diets based on animal products. As a result, some people believe that switching to a plant-based diet will benefit both their health and the environment. Additionally, consuming more plant-based foods can help control total calorie intake and lower the energy density of the diet. According to a study by Perignon et al. conducted in France, about one-fifth of French adults consumed a sustainable diet that was high in nutrition, which resulted in a 20% reduction in greenhouse gas emissions without any additional expense. The findings indicate that by making wiser dietary decisions, such as consuming less meat and alcohol, more plant-based foods, and moderate amounts of nutrients, it is possible to decrease greenhouse gas emissions and improve nutritional adequacy. It is unclear, though, how a healthy diet and low greenhouse gas emissions are related. It is claimed that in order to increase preference for sustainable diets, sustainable nutrition guidelines should include both nutritional and environmental considerations (Perignon et al., 2016). When an average diet in England is regulated in accordance with WHO recommendations, it has been demonstrated in an

epidemiological study by Gordon et al. there is a decrease in greenhouse gas emissions. Diets that are too restrictive may also limit consumption of some nutritious foods, such as fruit, which may limit their ability to have a positive impact on health. Instead, it is suggested that changes should be made to consume more grains, fruits, and vegetables while consuming less meat and processed food overall. With the exception of rice, cereals have relatively low greenhouse gas emissions (due to irrigation requirements and high methane gas production). The ecosystem is harmed by the use of irrigation, fertilizers, and pesticides on cereal crops. Whole grains and refined grains differ in their environments depending on certain nutrients. Cooking time and consumption of household energy can be decreased through refining (such as brown and white rice) (Gordon et al., 2018). Compared to white bread, whole wheat bread has a lower carbon footprint. Although there are differences in the nutritional balances, they are negligible. Grain consumption may have a lower environmental impact than other food groups. According to Eatwell's recommendations, more consumption of whole-grain foods should be encouraged due to their advantages for human health as well as the fact that they have less of an environmental impact on current production systems than other food groups. Low nutritional value foods with a lot of sugar and fat can lead to obesity and chronic diseases. Complex environmental effects result from it. Despite having low greenhouse gas emissions, sugary foods have the potential to degrade habitats and stress water supplies. Coffee and cocoa consumption is linked to habitat loss. These foods are not nutritionally prioritized and are merely personal preferences, so eating too much of them can result in wasteful greenhouse gas emissions, water waste, and land use. Different greenhouse gas emissions and land uses can be seen when examining each food group separately, and these variations are caused by the methodologies used. For instance, it is possible to identify fruits and vegetables with high and low greenhouse gas emissions, but cutting back on meat and milk consumption has a greater positive impact on the environment than opting for produce with low emissions. Food groups may have different environmental effects depending on where they are produced because production productivity affects the environment (E. Masset, Haddad, Cornelius, & Isaza-Castro, 2012; Perignon et al., 2016).

In a study conducted in France by Drewnowski et al. on a total of 483 different foods, greenhouse gas emissions were looked at. These 483 nutrients, which include grains, sugar, frozen and processed fruits and vegetables, meat and meat products, milk, and dairy products, are broken down into five basic food groups. Then, it was looked at how much each of these groups contributes to the emissions of greenhouse gases per 100 g and per 100 kcal. The two food groups with high density and lower nutrient con-

tent, such as sugar and cereals, were found to have the lowest greenhouse gas emissions in terms of both grams and kcal. Higher nutritional value meat and dairy products have the highest emissions of greenhouse gases per 100 grams and the lowest values per 100 kcal. Low greenhouse gas emissions are seen in the fruit and vegetable groups at 100 grams, but when 100 kcal values are taken into account, high greenhouse gas emissions are seen from storage. The general consensus was that nutrient-dense foods produce more greenhouse gas emissions per 100 kcal(Table 1.) (Darmon & Drewnowski, 2015).

Healthy eating and eating with little impact on the environment are not the same thing. It is important to eat a variety of foods as part of a healthy diet. However, a variety of foods with different nutrient contents are linked to greenhouse gas emissions. For instance, both animal and plant foods should be consumed to supply protein in a healthy diet, but the choices made will vary in terms of greenhouse gas emissions. As a result, a diet that is nutritionally sound need not be one that produces few greenhouse gas emissions. This is because certain foods have a greater dependence on greenhouse gas emissions (G. Masset, Soler, Vieux, & Darmon, 2014).

Table 1. Contribution of different food groups to total greenhouse gas emissions

Food products group	**Total Greenhouse Gas Emissions**
Meat, meat products and fish	%28
Dairy products	%23
Bread, biscuits, cake, flour	%13
Potatoes, fruits and vegetables	%15
Oils and fats	%3
Beverages and sweetened products	%15
Other nutrients	%3

Sustainable Nutrition Models

Mediterranean diet

Since studies began in the 1970s, the Mediterranean diet has been linked to lower disease risks. Mediterranean diet, which emphasizes plant-based foods such as fruit, vegetables, grains, legumes, seeds, whole-grain bread, nuts, and fish as well as nuts and olive oil, which are sources of monounsaturated fatty acids and antioxidants. Moderate amounts of red wine can also be consumed. It is a dietary pattern linked to low consumption of

foods high in trans fats, such as desserts and sweets. Phytochemicals and vitamins are also abundant (Elliot M Berry, 2019).

The various food cultures in the Mediterranean region can be seen in the Mediterranean diet as a whole. There is no single Mediterranean-style diet; instead, it has been customized to fit the cultures of the various nations. As a result, it represents various gastronomic and lifestyle traditions in the Mediterranean. Since the beginning of the 1990s, the pyramid nutrition model has gained popularity as a nutritious Mediterranean diet (Jones et al., 2016).

Due to the Mediterranean diet's plant-based foundation and lower greenhouse gas emissions than the Western diet, studies on its environmental sustainability have recently increased. The Mediterranean diet includes a wide variety of foods, including olive oil, fish, fruit and vegetables, legumes, and fermented milk, all of which are known to have health benefits, as well as a commitment to culture and tradition, respect for human nature, and a focus on seasonality and fruit and vegetables (J. L. Johnston, J. C. Fanzo, & B. Cogill, 2014). Due to factors such as low consumption of animal products and minimal environmental impact, it is regarded as being partially sustainable. The sustainability of food systems is jeopardized by a propensity to grow plants for economic gain, intensive farming practices, greenhouse farming, high meat consumption, and industrial food production (Meybeck & Gitz, 2017).

Double pyramid model

The double pyramid model was developed in 2009 and the most important innovation it brings is the relationship between the production and consumption of foods and the environmental effects of their diet. It is possible to implement environmentally sustainable diets without adversely affecting the economy with the dietary patterns recommended by experts such as the Mediterranean type diet. At the same time, the goals of public health and ecosystems converge (Jennie I Macdiarmid et al., 2012). Consuming every food in moderation, reducing the consumption of meat and dairy products, and increasing the consumption of fruits and vegetables provide benefits to the environment as well as to human health. The double pyramid model emerged to explain the environmental effects of food preferences. It is a diagram in which a new inverted 'environmental' pyramid is placed next to the classical food pyramid (Mediterranean diet) by classifying the ecological footprints of foods. The food pyramid shows the relationship between food products and nutritional values according to Mediterranean type nutrition principles. The environmental pyramid also shows the relationship between food products and their environmental effects. The pyramid on the left is based on the Mediterranean diet, which

has been designated by the FAO as an exemplary model of sustainable nutrition thanks to its healthy, affordable and low environmental impact (Figure 1) (Eker, Reese, & Obersteiner, 2019). Good adherence to a Mediterranean diet is associated with several health benefits, such as a reduction in the overall mortality rate and incidence of cardiovascular disease. This model of nutrition; emphasizes that it should be based on foods of plant origin as it provides vitamins, minerals, complex carbohydrates and fiber, and that the saturated fat, sugar and salt at the top of the pyramid should be consumed less. The pyramid on the right reclassifies nutrients by environmental impact, the top of which is an inverted pyramid containing the most environmentally harmful nutrients, largely reflecting the order of nutrients in the food pyramid. Life cycle assessment was used to measure the environmental impact of nutrients. Life cycle assessment; It is a technique that takes into account the energy consumption and environmental burden of the entire production system (Burlingame & Dernini, 2010). Results are described by three different indicators:

- Carbon footpuates equivalents by quantity,"
- Water footprint; total fresh water consumed to produce specific nutrients refers to the amount of
- Ecological footprint; Consider the various ways environmental resources are used.

It is an indicator that measures the anthropogenic impact by taking the Ecological footprint was used as a reference while creating the environmental pyramid. The reason for using this indicator is that it takes into account many environmental factors at the same time. At the same time, its unit of measurement was found to be easier to visualize and understand when compared to other indicators (Verma, Vishal, Kohli, & Kumar, 2021).

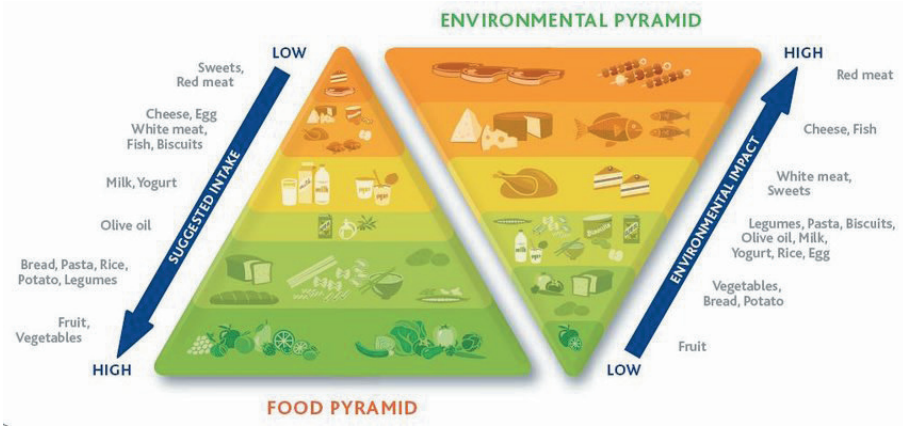

Figure 1. *Double pyramid model (Nelson, Hamm, Hu, Abrams, & Griffin, 2016)*

DASH (Dietary Approaches to Stop Hypertension) diet

The DASH diet's advantages for health are crucial for its effects on the environment as well. They can effectively reduce their nutrient consumption and greenhouse gas production by following the DASH diet and other plant-based eating plans. The DASH diet may be more widely accepted because of its potential benefits for public health and the environment, but there may still be some obstacles in the way of its adoption (Trautwein & McKay, 2020). Adoption of the DASH diet can be influenced by food costs in particular. Consuming foods that are suitable for the DASH diet was linked to higher costs in a study carried out in the United States. This study supported earlier research showing higher costs for healthier diets. Promoting affordable, healthy diets is a crucial step in ensuring that dietary advice is followed (Mendoza-Vasconez, Landry, Crimarco, Bladier, & Gardner, 2021).

New Nordic eating habits

The New Nordic Diet was created as a result of the recent success of the "New Northern Cuisine," and it consists of Scandinavian foods that have received high marks for flavor, sustainability, and health. Mithril et al. described the New Nordic Diet's guiding principles, which are explained in several recommendations (Mithril et al., 2012):

• Scandinavian origin; • It is based on dishes made with high-quality organic food products and has a rich cultural heritage. • Gastronomic potential. Vegetable foods like cabbage, legumes, potatoes, and herbs must have a variety of colors and flavors to complement the flavor of Arctic seafood and to help define Scandinavia.

Health; In contrast to many western nations, including Denmark, there is a lower consumption of meat and a higher consumption of seafood and plant-based foods like fruits, vegetables, and legumes.

Dietary elements should support physical, mental, and social wellbeing in addition to helping to prevent diseases like diabetes and cancer.

Sustainability; Using local foods to reduce the amount of nutrients transported, using organic products and foods from rural areas, swapping out meat group foods with plant foods, and minimizing food waste are all practices that should help lessen the environmental effects associated with food production (Mithril et al., 2012).

Vegan and vegetarian food

Transitioning to a vegetarian or vegan diet is not specifically advised in the American Dietary Guidelines for Americans. Because it is impossible to say for sure whether a vegetarian diet reduces health risks more than

increasing the consumption of plant foods in an omnivorous diet, which includes both meat and plant foods. The environmental impact of vegetarian meals and diets is lower than that of omnivorous diets. However, foods like meat, fish, and dairy are excellent sources of certain essential nutrients, so reducing your intake of these foods could make your health problems worse(González-García, Esteve-Llorens, Moreira, & Feijoo, 2018).

Effects of organic nutrients on greenhouse gases

The term "organic food system" is still relatively new and is evolving quickly. It began in Central Europe and has since spread almost everywhere in the world. The organic system is a global nutrient system today. In terms of nutrients, the term "organic" refers to the practices that go into producing food in accordance with these practices (Tilman & Clark, 2014). A food system supported by international standards and laws is represented by organic agriculture and food production. Legal definitions in Europe, Japan, and the United States include rules and a thorough certification process at both the national and private standards level. There are organic laws in many different nations. measurements of evaluations using metrics, indicators, and parameters. In Europe, it is connected to a sustainable and wholesome food system and the organic logo (E. M. Berry, 2019).

Conclusion

Although the concept of sustainable nutrition is not a new concept for developed countries, it is a new concept for our country. Public awareness should be raised on issues such as local and seasonal nutrition, reducing the consumption of animal products, and obtaining more efficient food, which are important in ensuring sustainable nutrition.

- Nutrition guidelines on the effects and practices of sustainable nutrition should be developed.
- One of the important issues in sustainable nutrition is to reduce meat consumption. However, meat consumption still has an important place in food patterns in our country and in the world. For this reason, more studies should be conducted on balanced and acceptable nutrition models in which greenhouse gas emissions are reduced to an acceptable extent.
- Food wastes and losses in homes are also an important issue for sustainable nutrition. Food waste leads to greenhouse gas emissions produced in vain. Individuals should prepare as much food as they eat and be more conscious of avoidable waste.
- Studies should be carried out to reduce and prevent nutrient wastes and losses.

- The study was conducted on adults, but the knowledge levels of younger people on sustainable nutrition should also be evaluated.
- Inclusion of courses on sustainability and sustainable nutrition in schools, and the introduction of various studies, competitions and projects on the subject can contribute to environmental protection by raising awareness at a young age.

Food preferences and dietary patterns of society and individuals can contribute to the creation of a sustainable diet. Local producers and local consumers can be supported and various related projects can be carried out.

- Sustainable food intake and sustainable preparation should be given importance.
- Seasonal consumption of foods should be encouraged by relevant institutions and organizations (eg Ministry of Health, Ministry of Food, Agriculture and Livestock).
- Various projects can be carried out together with lawmakers and related institutions in order to develop new policies on sustainable nutrition.
- Studies on sustainable nutrition in our country and in the world are limited. More extensive and detailed studies on this subject are required.
- In our country, as in other countries, there is a need for studies in which the greenhouse gas emission amounts of foods are calculated and the effects of dietary habits on greenhouse gas emissions are measured.

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