

**EXAMINATION OF THE EFFECTS OF DIETARY MIRNAS ON THE
DEVELOPMENT OF FOOD ALLERGY AND OF FOOD-DERIVED
NANOVESICLES ON A HUMAN BASOPHIL CELL LINE**

by

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ABSTRACT

EXAMINATION OF THE EFFECTS OF DIETARY MIRNAS ON THE DEVELOPMENT OF FOOD ALLERGY AND OF FOOD-DERIVED NANOVESICLES ON A HUMAN BASOPHIL CELL LINE

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basophil cell line

Food allergy is a severe, aberrant immunological reaction to specific food proteins caused by the immune system misinterpreting the food protein as dangerous. Food allergies can range in severity from mild to life-threatening, affecting various parts of the body and causing common symptoms such as fatigue, swelling, shortness of breath, gastrointestinal distress, vomiting, and in extreme cases anaphylaxis. The most common type of food allergy is IgE-dependent, often known as Type I immediate hypersensitivity. Despite the prevalence of food allergies, no safe gold standard for diagnosis or effective treatment is currently available. The only option to avoid allergic responses to food is a strict elimination diet, which is nearly impossible to follow for the rest of one's life owing to industrialization, and an epinephrine injection, which must be administered immediately and necessitates critical decision-making on the part of the patient and/or persons nearby. It causes social anxiety and significantly reduces the patient's, family's, and friends' quality of life. Better diagnostic procedures and effective therapies for food allergies are required. Our research explores the role of dietary microRNAs in the development of food allergy reactions. We propose that miRNAs encapsulated in lipid nanovesicles may migrate deeper into our gastrointestinal system, be ingested, and trigger immunomodulation through gene expression regulation, leading to allergic responses. To test our hypothesis, we collect RNA, miRNA, and nanovesicles from peanuts and eggs, two of the most prevalent allergenic foods. Gel electrophoresis and spectrophotometry are two procedures used to analyze the quality, purity, and concentration of nucleic acids. To characterize nanovesicles, spectroscopy, nanovesicle tracking analysis, and scanning electron microscopy are employed, and where appropriate, nanovesicle samples are purified using a sucrose cushion protocol combined with TCA precipitation. We cultured

a KU812 human basophil cell line to observe the functionality of nanovesicles on degranulation and activation, using CD63 and CD203c antibodies in flow cytometry. Conserved miRNAs that may influence allergy genes are identified using three different miRNA-targeting tools, miRanda, RNAhybrid, and TargetScan. Furthermore, RNA-seq analysis is used to determine the most prevalent miRNAs. Our research on the role of miRNAs in food allergies has the potential to greatly increase our understanding of this complicated disease and lead to the development of better diagnostic approaches and treatments.



ÖZET

GIDASAL MİRNALARIN BESİN ALERJİSİ GELİŞİMİNE KATKISININ İNCELENMESİ VE BESİN NANOVEZİKÜLLERİNİN İNSAN BAZOFİL HÜCRE HATTINA ETKİLERİNİN BELİRLENMESİ

ZEYNEP NUR EGLENEN

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Tez Danışmanı: Dr. Öğr. Üyesi Stuart James Lucas

Anahtar Kelimeler: besin alerjisi, besinsel miRNA, eksozom, küçük RNA sekanslaması,
KU812 insan bazofil hücre hattı

Besin alerjisi, bağışıklık sisteminin gıda proteinini tehlikeli olarak yanlış yorumlaması nedeniyle ortaya çıkan, belirli gıda proteinlerine karşı şiddetli, anormal bir immünolojik reaksiyondur. Besin alerjileri, vücudun çeşitli bölgelerini etkileyen ve yorgunluk, şişme, nefes darlığı, mide-bağırsak rahatsızlığı, kusma gibi yaygın, hafif semptomlara ve ekstrem durumlarda anafilaksi gibi yaşamı tehdit eden reaksiyonlara neden olabilir. En yaygın besin alerjisi türü, genellikle tip I aşırı duyarlılık olarak da bilinen IgE aracılı besin reaksiyonlarıdır. Besin alerjilerinin yaygınlığına rağmen, şu anda teşhis veya etkili tedavi için güvenli bir altın standart mevcut değildir. Yiyeceklere karşı alerjik tepkilerden kaçınmanın tek yolu, sanayileşme nedeniyle kişinin hayatının geri kalanında uygulaması neredeyse imkansız olan katı bir eliminasyon diyeti ve hemen uygulanması gereken ve hasta ve/veya yakınlarıdaki kişiler tarafından kritik kararlar almayı gerektiren epinefrin enjeksiyonudur. Bunlar sosyal anksiyete doğurur, ve hastanın, ailesinin ve arkadaşlarının yaşam kalitesini önemli ölçüde düşürür. Besin alerjileri için daha iyi teşhis prosedürleri ve etkili tedaviler gereklidir. Araştırmamızda besin alerjisi reaksiyonlarının gelişiminde gıdasal mikroRNA'larının rolünü araştırmaktayız. Lipid nanoveziküller içinde korunmuş miRNA'ların gastrointestinal sistemimizde daha derinlere taşınabileceğini, sindirilebileceğini ve gen ekspresyonu düzenlemesi yoluyla immünomodülasyonu tetikleyerek alerjik tepkilere yol açabileceğini düşünüyoruz. Hipotezimizi test etmek için, en yaygın alerjenik gıdalardan ikisi olan yer fıstığı ve yumurtadan RNA, miRNA ve nanovezikül izole ettik. Jel elektroforezi ve spektrofotometri ile nükleik asitlerin kalitesini, saflığını ve konsantrasyonunu analiz ettik. Nanovezikülleri karakterize etmek için spektrofotometri, nanovezikül izleme analizi ve taramalı elektron mikroskopu kullandık, ve gerektiği yerlerde nanovezikül örneklerini süroz tampon metodu ve TCA çöktürmesi yöntemleriyle saflaştırdık. Akış sitometrisinde CD63 ve CD203c antikoru ile nanoveziküllerin degranülasyon ve aktivasyon üzerindeki işlevselliğini

gözlemlemek için KU812 insan bazofil hücre hattını kullandık. Alerji genlerini etkileyebilecek korunmuş miRNA'ları tanımlamak amacıyla, üç farklı miRNA-hedef aracı, miRanda, RNAhybrid ve TargetScan, kullandık. Ayrıca, bu gıdalardeki en yaygın miRNA'ları belirlemek için RNA-seq analizi yaptık. Besin alerjilerinde miRNA'ların rolüne ilişkin araştırmamız, bu karmaşık hastalıktaki güncel anlayışımızı büyük ölçüde artırma ve daha iyi teşhis yaklaşımları ve tedavilerin geliştirilmesine yol açma potansiyeline sahiptir.



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To my family...

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LIST OF ABBREVIATIONS

AGO	Argonaute
AGO2	Arganaute2
APC	Antigen-Presenting Cell
ATP	Adenosine triphosphate
BAT	Basophil Activation Test
bp	Base pair
DBPCFC	Double-blind placebo-controlled food challenge
DEG	Differentially Expressed Gene
DGCR8	DiGeorge Syndrome Critical Region 8
EHT	Extra High Tension
EPIT	Epicutaneous immunotherapy
FACS	Flow cytometry staining buffer
FITC	Fluorescein isothiocyanate
GC	Guanine-cytosine
HDL	High-density lipoprotein
IAA	Isoamyl alcohol
IEC	Intestinal epithelial cell
IgE	Immunoglobulin E
M-cell	Microfold cell
MFI	Mean fluorescence intensity
MHC II	Major histocompatibility complex II
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MREs	miRNA response elements
mRNA	messenger RNA
NPM1	nucleophosmin1
Nt	nucleotide
NTA	Nanovesicle Tracking Analysis
NV	Nanovesicle
OFC	Oral Food Challenge

OIT	Oral immunotherapy
PBS	Phosphate Buffered Saline
PE	phycoerythrin
PEG	Polyethylene glycols
pre-miRNAs	precursor miRNAs
pri-miRNAs	primary miRNAs
psIgE	peanut-specific IgE
PTAH	Peanut Allergen Powder-dnfp
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription polymerase chain reaction
SCIT	Subcutaneous immunotherapy
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscopy
SGF	Simulated gastric fluid
SGWS	Sucrose gradient working solution
sIgE	specific Ig-E
SLIT	Sublingual immunotherapy
SPT	Skin Prick Test
SRA	Sequeunce Read Archive
TCA	Trichloroacetic acid
Th2	T helper cell 2
TSLP	thymic stromal lymphopoietin
UTR	Untranslated region
v/w	volume/weight
WD	Working Distance

LABORATORY DEVICES AND KITS

Cell Freezing: Thermo Scientific™ Nalgene™ Mr. Frosty® freezing container

Cell hood: Thermo Scientific HERASAFE KS 12

Centrifuges: Eppendorf 5418 R, Beckman Coulter Microfuge 16; Eppendorf Centrifuge 5424 R; VWR® Mega Star 3.0R

Flow Cytometry Antibodies: PE-conjugated CD203c anti-human antibody (clone: NP4D6; eBioscience), FITC-conjugated CD63 anti-human antibody (MEM-259, Invitrogen)

Flow cytometer: BD LSRFortessa™ Cell Analyzer

Fume hood: Wesemann Delta System 30

Gel imager: BIO-RAD Gel Doc EZ Imager

Hemocytometer: Thermo Fisher Scientific Countess II Automated Cell Counter

Image lab software: BIO-RAD Image Lab 6.0

Microscopy: Zeiss Primovert Inverted Light Microscope

Nanodrop: NanoDrop 2000c UV Spectrophotometers by Thermo Scientific

Nanovesicle tracking analysis: Malvern Panalytical NanoSight NS300

pH meter: Thermo Scientific Orion Star A211 pH meter

SEM: Zeiss Supra 35VP field emission scanning electron microscope (FESEM)

Thermostat: Eppendorf ThermoStat Plus

Transilluminator: Vilber Lourmat ECX-F20.M V1

Vortex: Cole-Palmer™ Stuart Variable Speed SA8 Vortex Mixer; Mixer UZUSIO VTX-3000L

Water bath: Stuart SW

1. INTRODUCTION

1.1 Epidemiology

Food allergies are caused by an inappropriate immunological response to certain dietary proteins. When a person with a food allergy consumes or comes into contact with an allergenic food, their immune system incorrectly recognizes the food protein as harmful and initiates an immunological reaction. This immune reaction results in the secretion of allergen-specific antibodies, typically immunoglobulin E (IgE), which cause the release of substances such as histamine, resulting in a variety of allergy symptoms. Severity of food allergies can range from mild to severe, and might affect different parts of the body, including the skin, gastrointestinal tract, respiratory system, and cardiovascular system (Waserman et al., 2018). Common symptoms of a food allergy may include fatigue, swelling, shortness of breath, urticaria, gastrointestinal distress, vomiting, muscle pain and in severe cases, anaphylaxis, a potentially fatal reaction requiring immediate medical intervention (Żukiewicz-Sobczak et al., 2013).

Although specific foods that cause allergies vary from person to person, according to the recent study of Health Canada, cow's milk, egg, peanut, tree nuts, fish/shellfish, wheat, sesame seed, soy, mustard and sulphites are among the most common food allergens. Individuals with food allergies need to carefully manage their diet and be aware of potential allergens to avoid allergic responses (Waserman et al., 2018).

1.1.1. Prevalence

The incidence of food allergies varies according to the population investigated and the allergens under consideration. Indeed, determining food allergy prevalence with certainty is incredibly difficult due to a plethora of factors and methodological limitations. However, research has revealed that food allergies are becoming more widespread, particularly among children. The exact causes of the rising prevalence of food allergies are not yet fully understood. However, various explanations have been proposed by researchers to explain this pattern such as the hygiene hypothesis, delayed introduction of allergenic foods, changes in

dietary habits, environmental and epigenetic factors (Prescott & Allen, 2011). In general, it is estimated that around 2-10% of the population worldwide is affected by food allergies (Loh & Tang, 2018).

The prevalence of food allergies can vary significantly between different countries and regions. For example, Western countries, such as the United States and the United Kingdom, tend to have higher rates of food allergies compared to developing nations. However, recent research suggests that the prevalence is increasing in developing countries as well (Loh & Tang, 2018).

While many children outgrow their food allergies, some allergies persist into adulthood. For example, allergies to peanuts, tree nuts, fish, and shellfish are more likely to remain throughout life. In contrast, allergies to milk, eggs, soy, and wheat are more commonly outgrown (Sicherer et al., 2020).

1.1.2. Risk Factors

Several risk factors have been identified for the development of food allergies. While having one or more risk factors improves one's chances of acquiring a food allergy, it does not ensure that one will get one. The interplay between genetics, environmental factors, and immune responses is complex and still not fully understood.

Allergies have a genetic component, and people who have allergic family members are more vulnerable (Gerrard et al., 1976; Misiak et al., 2009). A family history of allergies, especially among direct family members such as parents or siblings, increases the likelihood of developing a food allergy. A survey with 622 adults and children reported that a child's risk of developing peanut allergy is increased sevenfold if he or she has a parent or sibling who is allergic to peanuts (Hourihane et al., 1996). Relatedly, individuals who have a personal or family history of allergic disorders such as asthma, eczema, or allergic rhinitis are more likely to develop food allergies. This suggests a shared underlying allergic tendency (Turnbull et al., 2014).

Delaying the introduction of potentially allergenic foods during infancy beyond the recommended time has been linked to an increased risk of developing food allergies. According to a seven-year study, the longer a child is breastfed, the greater the incidence of

atopic eczema. Early introduction, especially in the presence of breastfeeding, may help induce tolerance to these foods (Bergman et al., 2002).

Environmental factors may play a role in the development of food allergies. Repeated exposure to allergens such as pollen, dust mites and pollution may increase the risk of sensitization and allergy development. Changes in the gut microbiota due to antibiotic use, dietary changes, consumption of processed foods or a lack of microbial diversity may also modulate the immune reactions. The "hygiene hypothesis" suggests that reduced exposure to infectious agents and certain microorganisms in early childhood as a result of improved sanitation and smaller family sizes may impact immune system development and may contribute food allergy (Lack, 2008).

Certain other factors like being born via caesarean section, lack of vitamin D, being of certain ethnic backgrounds, and even certain seasons of birth have been associated with an increased risk of food allergies (Lack, 2008; Matsui et al., 2019). However, further research is needed to fully understand their role.

1.2 Why Does Food Allergy Arise?

1.2.1 Molecular Basis

The molecular basis of food allergies involves a complex interplay between the immune system, specific allergenic proteins in food, and the individual's genetic predisposition. It is a group of clinicopathological conditions, not a single disease (Anvari et al., 2018). There are four types of food allergy categorized according to the unique immunological processes involved: IgE-mediated, non-IgE-mediated, mixed IgE- and non-IgE-mediated food allergies, and oral allergy syndrome.

Examples of allergenic dietary proteins include for example casein in milk, ovalbumin in eggs and cupins in peanuts (Mueller et al., 2014; Dantas et al., 2017 & Docena et al., 1996). These proteins have specific structural characteristics that allow them to interact with the immune system and trigger an allergic response in susceptible individuals. They are resistant to heat, enzymes and low-pH conditions which makes them stable throughout the gastrointestinal tract (Moriyama, 2015).

The most common form of food allergy is the IgE-dependent type, also known as immediate type I hypersensitivity. The development of a food allergy typically begins with a process known as sensitization. During sensitization, inflammatory cytokines such as interleukin-25 (IL-25), interleukin-33 (IL-33) and thymic stromal lymphopoietin (TSLP) are released, activating one of the antigen-presenting cells (APC), dendritic cells, into phenotypes normally acquired during pathogen defense. These activated dendritic cells then stimulate naive T cells, causing them to develop a T helper cell 2 (Th2) phenotype, which in turn promotes inflammatory signals. B and T lymphocytes recognize these proteins as allergens, activate, multiply, and differentiate, consequently producing food antigen-specific IgE (Anvari et al., 2019).

In individuals with food allergies, upon subsequent exposure to the allergenic protein, it binds to specific IgE antibodies that are already bound to mast cells and basophils. This binding leads to the activation of these cells and the release of various chemical mediators, such as histamine, tryptase, platelet activating factor, prostaglandins, and leukotrienes. The release of these chemical mediators triggers an inflammatory response, resulting in the characteristic symptoms of a food allergy (Figure 1.1) (Schoos et al., 2020).

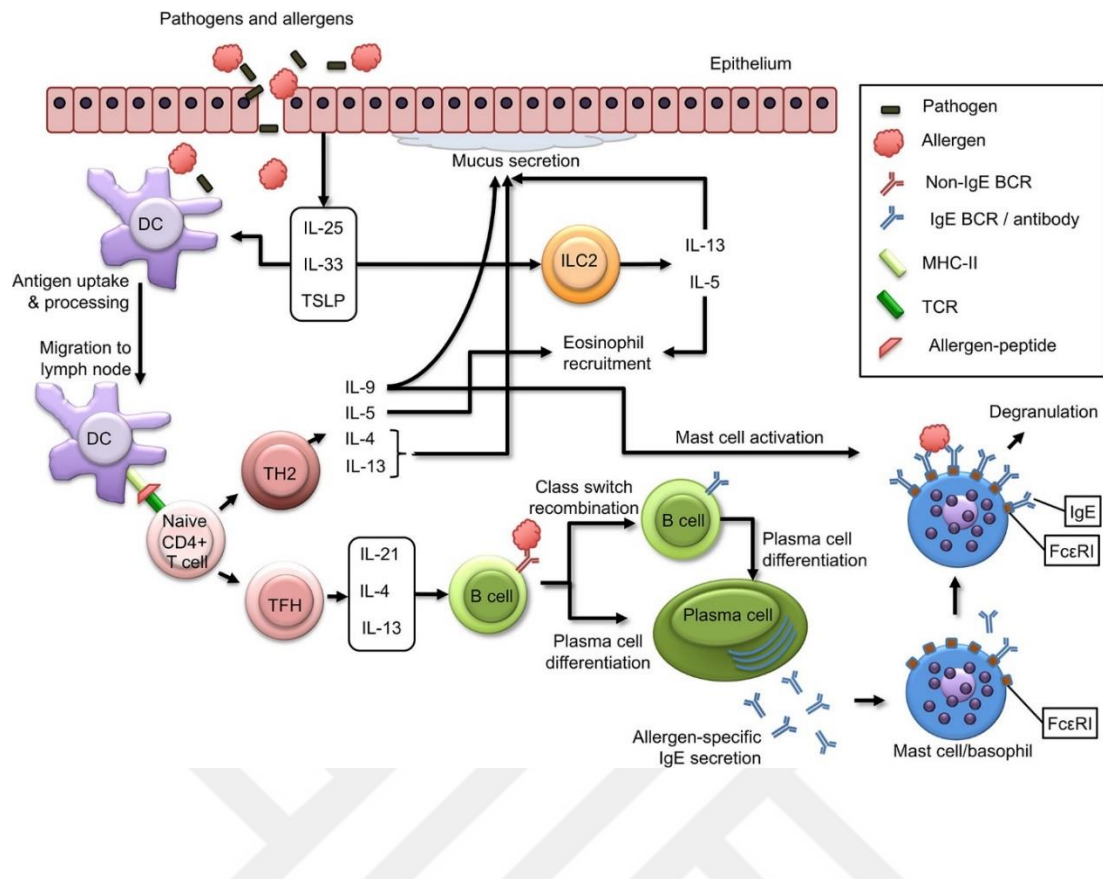


Figure 1.1: Molecular mechanisms of sensitization in allergy (Schoos et al., 2020). IL-25, IL-33 and TSLP are produced by epithelial cells due to contact with the allergen. These alarmins stimulate dendritic cells to generate TH2 responses and trigger TFH cells. IL-24, IL-4 and IL-13 secreted by TFH cells stimulate B cells to generate allergen-specific IgE. These IgEs make complex by binding to FcεRIs on mast cells and basophils, which can crosslink when re-exposed to the allergen.

1.2.2 Role of Basophils

Basophils are granulocytes, a type of white blood cell that plays a role in the immune system's reaction to allergens and parasitic infections. They are produced from bone marrow cells and circulate in the bloodstream. They are distinguished by the presence of granules carrying a variety of chemical mediators, including histamine and heparin. They are relatively rare in circulation, constituting only a small percentage of the total white blood cell population.

Basophils play a role in allergic reactions, especially acute hypersensitivity reactions. When exposed to an allergen, basophils become activated due to the binding of allergen-specific IgE antibodies to their surface. This causes the granule contents, including histamine, to be released, contributing to the symptoms of an allergic reaction (Siracura et al., 2013). Histamine is a potent vasodilator that promotes blood vessel dilation and increased permeability, contributing to the characteristic symptoms of food allergy, such as itching, urticaria, and swelling. Other mediators released by basophils can further amplify the allergic response and recruit other immune cells such as eosinophils to the site of the allergic reaction (Kabashima et al., 2018).

Basophils can also interact with other immune cells, such as T cells and dendritic cells, to promote and amplify the immune response in food allergies. They can secrete cytokines, such as interleukin-4 (IL-4) and interleukin-13 (IL-13), which are involved in the development of allergic responses and the differentiation of immune cells (Redrup, 1998).

1.3 Therapeutic Approaches in Food Allergy

1.3.1 Diagnosis

The diagnosis of food allergies involves a combination of medical evaluation, clinical history assessment, and specific diagnostic tests such as oral food challenge (OFC), skin prick test (SPT), basophil activation test (BAT), blood Ig-level test and omics-based tests. Qualified healthcare professionals experienced in allergy diagnosis and management will consider the individual's clinical history, symptoms, and test results to make an accurate diagnosis and provide appropriate guidance for allergen avoidance and management strategies. Some unstandardized and unverified tests, such as basophil histamine release, electrodermal and intradermal testing should be avoided during the diagnostic process (Sicherer & Sampson, 2014).

A detailed medical history is crucial in identifying potential food allergies. The healthcare providers ask about symptoms experienced after consuming specific foods, the timing and duration of symptoms, and any known triggers. They also inquire about personal or family history of allergies, asthma, eczema, or other allergic conditions. For example, the onset of

atopic eczema within the first 6 months of life is linked to the emergence of food allergies to egg, peanut, and milk (Turnbull et al., 2014).

An elimination diet involves removing suspected allergenic foods from the diet to see if symptoms improve. This is typically performed under the guidance of a healthcare professional or registered dietitian to avoid nutritional deficiencies. After a period of elimination, foods are gradually reintroduced one at a time to identify the specific food causing the allergic reaction. Additionally, keeping a detailed food diary can help track symptoms and identify potential patterns or correlations between certain foods and allergic reactions. However, it should be noted that medical history is unreliable and open to misperceptions since it depends on the patient's recollection of the events (Sampson, 1999).

In some cases, an OFC may be conducted under medical supervision. The double-blind placebo-controlled food challenge (DBPCFC) is accepted as the gold standard for food allergy diagnosis which involves the controlled ingestion of the suspected allergenic food in increasing amounts to observe the development of allergic symptoms. However, due to the risk of anaphylaxis, the high cost, resource-intensive and time-consuming process, oral food challenges are practically used only in strictly necessary conditions (Turnbull et al., 2014).

SPT involves placing a small amount of allergenic extracts on the skin, usually the forearm or back. The skin is then gently pricked using a sterile lancet to allow the allergen to enter the top layers of the skin. If a person is allergic to the specific allergen, an allergic reaction will occur at the site, characterized by a raised bump or wheal (Schoos et al., 2020).

Blood tests, such as specific IgE (sIgE) tests or component-resolved diagnostics (CRD), measure the levels of allergen-specific IgE antibodies in the blood. These tests can provide an indication of sensitization to specific allergenic proteins. However, they cannot definitively diagnose a food allergy and are often used in conjunction with other diagnostic methods (Sicherer & Sampson, 2014).

1.3.2 Treatment

Currently, there are no medicines or treatments that can cure or protect against food allergies with the exception of Palforzia, an oral immunotherapy (OIT) agent for peanut allergy approved by Food and Drug Administration (FDA) recently. Other than that, the treatment of

food allergies involves a combination of strict allergen avoidance, education of families and patients to follow the guidelines, emergency preparedness, and, in certain circumstances, medical intervention (Hwang et al., 2022).

The primary strategy for managing food allergies is to strictly avoid the allergenic food or foods that trigger an allergic reaction. This involves reading food labels carefully, being mindful of hidden or cross-contaminated allergens, and taking precautions when dining out or eating at social events. Even so, according to a survey, almost 20% percent of children diagnosed with food allergy had to visit emergency departments due to food allergy related anaphylaxis each year in the United States (Gupta, R. et al., 2018). Unfortunately, this strategy causes anxiety in children and families, as well as social isolation at social gatherings, camps, celebrations, and sleepovers (Hwang et al., 2022).

Individuals with severe food allergies, especially those at risk of anaphylaxis, should be prepared for emergency situations. Carrying an epinephrine auto-injector, knowing when and how to use it, and having an emergency action plan in place are all part of this. Family members, friends, and caretakers should also be trained to recognize the signs of an allergic reaction and to deliver emergency treatment if necessary. Other medications may be prescribed to manage specific symptoms or provide relief during accidental exposure. Antihistamines can help alleviate mild to moderate allergic symptoms such as itching and hives, and corticosteroids reduce inflammation and suppress immune responses. However, it should be noted that these should not be relied on the food allergy management and priority should be given to the epinephrine injection (Sicherer & Sampson, 2014).

Immunotherapy is an emerging treatment option for some food allergies. Four types of immunotherapies currently under research are subcutaneous immunotherapy (SCIT), oral immunotherapy (OIT), sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT). They involve controlled exposure to small amounts of the allergenic food over time, gradually increasing the dose to desensitize the immune system (Hwang et al., 2022).

Palforzia by Aimmune is the first OIT drug that has been approved by FDA to treat peanut allergy in children. It is given on a daily basis and comprises Peanut Allergen Powder-dnfp (PTAH). Clinical trials reported that children who received 300 mg PTAH daily for two years had higher tolerance to peanut ingestion and lower levels of serum peanut-specific IgE (psIgE) (Fernandez-Rivas et al., 2021). Although Palforzia has been shown to be safe and

effective, 85% of participants suffered adverse effects in their gastrointestinal tract and 81% in their respiratory tract (Vickery et al., 2018).

1.4 Roles of miRNAs

1.4.1 miRNAs

MicroRNA (miRNA) is a class of small non-coding RNA molecules that are typically about 19-25 nucleotides long and play a crucial role in post-transcriptional gene regulation. They are involved in various biological processes, including development, cell differentiation, metabolism, apoptosis, and response to stress. Dysregulation of miRNAs has been implicated in several diseases, including cancer, cardiovascular disorders, neurological conditions, and immune disorders (MacFarlane et al., 2010). Because of their regulatory functions and potential as diagnostic and therapeutic targets, miRNAs have received significant attention in biomedical research.

miRNAs can inhibit translation or induce degradation of the targeted mRNA by binding to specific messenger RNA (mRNA) molecules, thereby regulating the expression of genes. This is a natural process known as RNA interference (RNAi), which has been conserved in many eukaryotes as a host defense against RNA viruses (Xu et al., 2019). Primary miRNAs (pri-miRNAs) are synthesized from miRNA genes in the nucleus. They have a hairpin structure and are approximately 70 nucleotides long. A microprocessor complex consisting of an RNA binding protein, DiGeorge Syndrome Critical Region 8 (DGCR8), and a ribonuclease III enzyme, Drosha, is required to cleave the pri-miRNA into precursor miRNAs (pre-miRNAs). As a result of this step, a 2 nt 3' overhang is formed on the pre-miRNA. Pre-miRNAs are produced and subsequently exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex before being processed by the RNase III endonuclease Dicer. Thus, the mature miRNA duplex is acquired through the removal of the terminal loop. Mature miRNAs are loaded onto Argonaute (AGO) proteins to form a miRNA-induced silencing complex (miRISC) (Figure 1.2). miRISC recognizes and binds to target mRNA through complementary sequences known as miRNA response elements (MREs) (O'Brien et al., 2018).

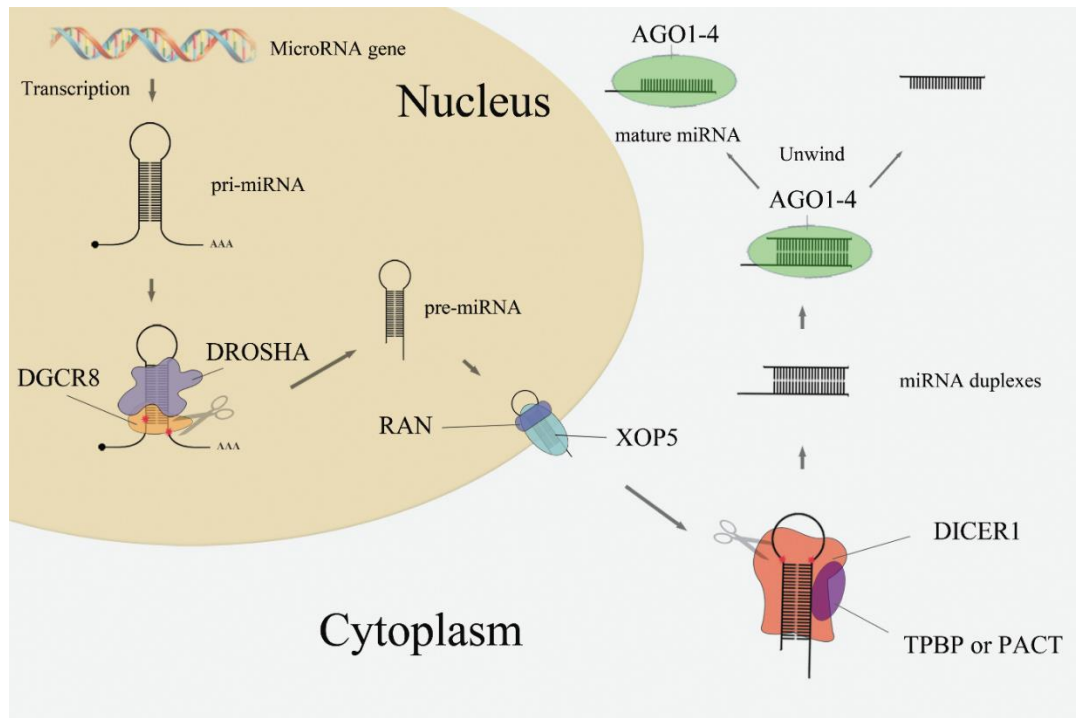


Figure 1.2: miRNA biogenesis pathway (He et al., 2016). Pri-miRNA is transcribed from miRNA gene in the nucleus. It is cut from both ends by DGCR8 and DROSHA complex to obtain pre-miRNA with 2 nucleotides 3' hang. It is exported to the nucleus with RAN and XOP5 protein complex. DICER1 complex removes the hairpin structure, then the miRNA duplex is loaded into the AGO protein. A functioning miRISC complex is complete when one strand is detached.

1.4.2 Extracellular/Circulating miRNAs

Endogenous miRNAs that circulate in the extracellular area are known as cell free-circulating miRNAs. They regulate gene expression and participate in a variety of biological processes as intercellular miRNAs (Cui et al., 2019). They are found in various body fluids, including blood, serum, tears, urine, saliva, and breast milk (Salloum-Asfar et al., 2019).

Depending on the mechanism of release, circulating miRNAs are categorized into vesicle-associated and non-vesicle-associated miRNAs (Figure 1.3). Non-vesicle-associated circulating miRNAs constitute the majority of the extracellular miRNAs such as ribonucleoprotein complex. Protective properties of protein miRNA carriers such as Arganoute2 (AGO2), GW182, nucleophosmin1 (NPM1), and high-density lipoprotein (HDL) have been confirmed with several studies. Alternatively, circulatory miRNAs can be

transferred to distant tissues by encapsulation in exosomes or microvesicles. This involves an active loading process that uses adenosine triphosphate (ATP) as its energy source and may be related with selectivity and a high order of regulation in homeostasis. Several studies have suggested that miRNAs protected and shuttled by exosomes play crucial regulatory roles in cell-cell communication (Cui et al., 2019).

MiRNA dysregulation has been linked to a variety of human disorders, including cardiovascular disease, diabetes, allergic rhinitis, atopic dermatitis, asthma, and cancer (Hamam et al., 2017; Weidner et al., 2020). Their extreme structural stability and high resistance against RNase degradation make circulating miRNAs suitable noninvasive diagnostic biomarker in several diseases (Mitchell et al., 2008).

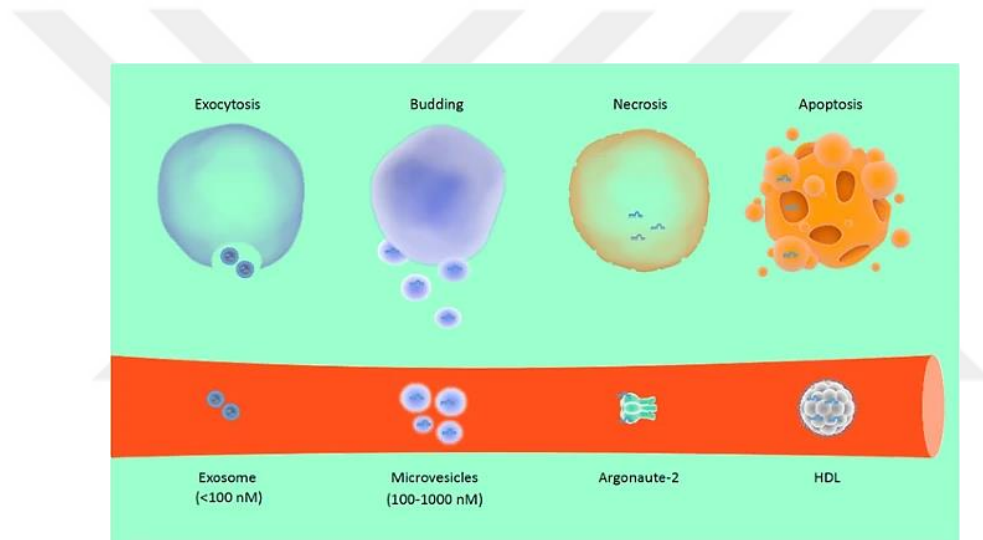


Figure 1.3: Carrying molecules of extracellular miRNAs and their formation mechanisms. (Hamam et al., 2017). Vesicle-associated extracellular miRNAs could be encapsulated in microvesicles or exosomes, while non-vesicle-associated ones could travel by binding to carrier proteins.

The study of the functions, mechanisms of action, and impacts of circulating miRNAs is still ongoing. Our present understanding, as summarized in Figure 1.4, is growing each year, bringing new insights into the nature of miRNAs and their potential applications in medical diseases (Salloum-Asfar et al., 2019).

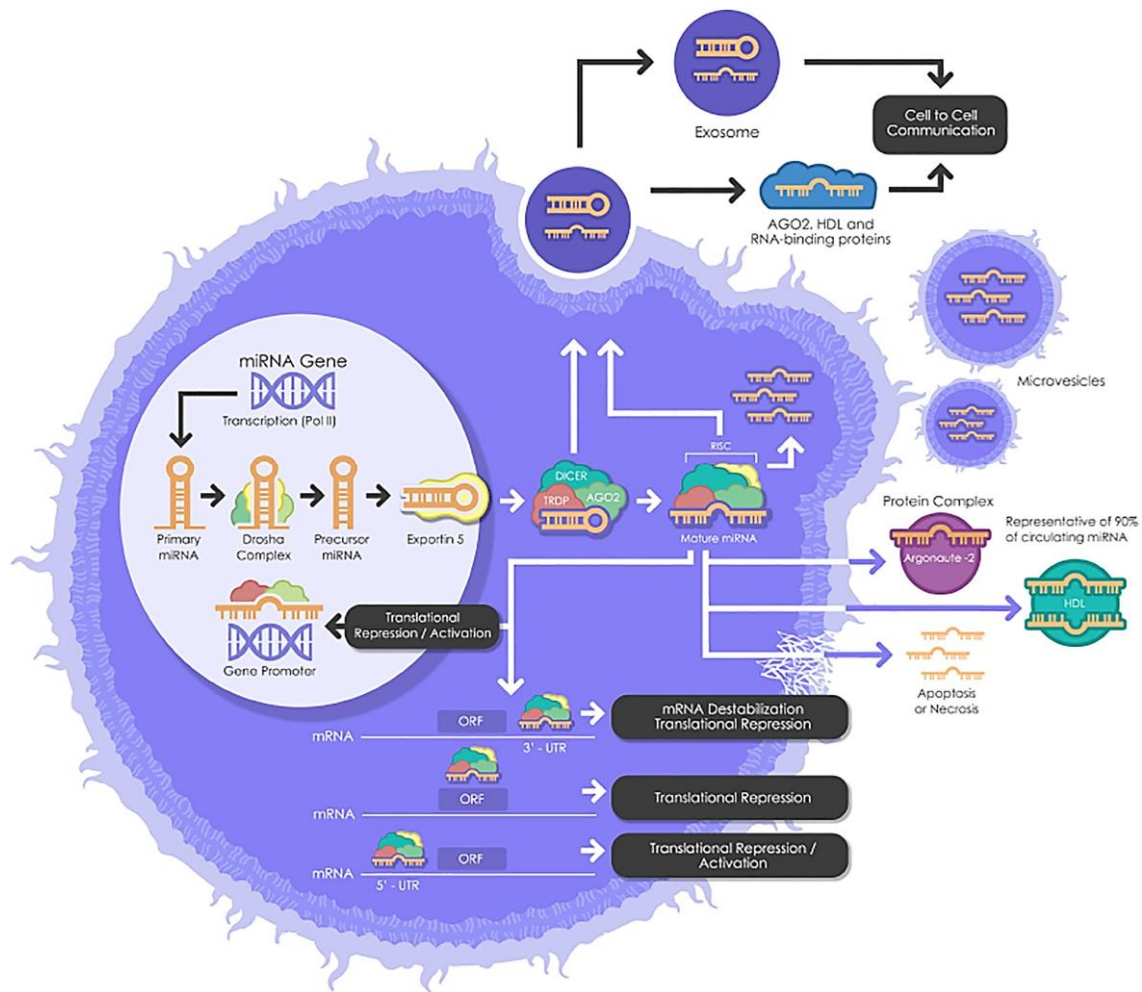


Figure 1.4: Summary of miRNA biogenesis, secretion pathways to the extracellular space, and biological function (Salloum-Asfar et al., 2019). miRNAs serve an important function in expression regulation by destabilizing mRNA and repressing/activating translation. They are modified by multiple protein complexes in the nucleus and in the cytoplasm. They can migrate to specific parts of the body, either by encapsulation inside vesicles or by adhesion to a carrier protein. As a result, they can modify the gene expression in distant tissues and coordinate cell-to-cell communication.

1. SCOPE OF THE THESIS

The objective of this study is to identify the importance of miRNAs in the allergic responses to food. Our hypothesis is that miRNAs protected inside food-derived nanovesicles modify gene expression and modulate allergic reactions when ingested by the host cells. To examine our proposal, we isolated RNA, miRNA and nanovesicle from the two most prevalent allergenic foods, peanut and egg. We used Illumina small RNA sequencing followed by an RNA-seq analysis to establish which miRNAs are most prevalent in our food sources. The consensus of three miRNA-target tools determined which human allergy genes could be targeted by peanut and egg miRNAs. Nanovesicle samples were purified and characterized by SEM, NTA and spectrophotometric analysis. Using CD63 and CD203c antibodies and the KU812 human basophil cell line, functional biological effects of dietary nanovesicles were evaluated in a flow cytometry setting.

2. MATERIALS & METHODS

3.1 MATERIALS

Table 3.1: Computational tools and databases used in the study.

Tools	Purpose	Source
Bash	running scripts, converting file formats	GNU
BLAST-plus	alignment	(Camacho et al., 2009)
Bowtie	alignment	(Langmead et al., 2009)
miRanda – 1.9	miRNA-target prediction	(Enright et al., 2003)
R	RNA-seq analysis, filtering, parsing, visualization	(R Core Team, 2023)
RNAhybrid	miRNA-target prediction	(Rehmsmeier et al., 2004)
TargetScan	miRNA-target prediction	(McGeary et al., 2019)
miRDeep2	miRNA alignment	(Friedländer et al., 2012)
ImageJ	Image processing	(Schneider et al., 2012)
Database	Purpose	Source
Sequence Read Archive (SRA)	downloading raw data	(Leinonen et al., 2011)
miRBase	miRNA sequences and annotations	(Kozomara et al., 2018)
ENSEMBL	<i>Homo sapiens</i> reference files for GRCh38.v.104	(Schneider et al., 2016)
Reactome	pathway browser	(Gillespie et al., 2022)
NCBI Genbank	<i>Arachis hypogaea</i> reference files for arahy.Tifrunner.gnm1.KYV3	(Schmutz et al., 2018)

Table 3.2: Chemicals used in the study.

Chemical Name	Purchased From
NaCl	Merck (New Jersey, USA) (CAS #: 7647-14-5)
EDTA	Sigma-Aldrich (Missouri, USA) (CAS #: 15708-41-5)
Tris	MP Biomedicals (California, USA) (CAS #: 1.800.854.0530)
β -mercaptoethanol	BioFroxx (Einhausen, Germany) (CAS #: 1414ML500)
Chloroform	Merck (Darmstadt, Germany) (CAS #: 67-66-3)
Isoamyl alcohol	EMD Millipore (Massachusetts, USA) (CAS #: 603-006-00-7)
Isopropanol	BioFroxx (Einhausen, Germany) (CAS #: 67-63-0)
Boric Acid	MP Biomedicals (California, USA) (Cat #: 194810)
PEG, average M_n 6000	Sigma-Aldrich (Missouri, USA) (CAS #: 25322-68-3)
LiCl	Sigma-Aldrich (Gillingham, UK) (CAS #: 7447-41-8)
Ethanol Absolute	Merck (Darmstadt, Germany) (CAS #: 64-17-5)
1X TE Buffer	Promega (Wisconsin, USA) (Cat. #: PAV6232)
Formamide	BioFroxx (Einhausen, Germany) (CAS #: 75-12-7)
Agarose	PeqGOLD (Erlangen, Germany) (Cat. #: 732-2788)
PBS	HyClone (Utah, USA) (Cat #: SH30256.01)
Proteinase K	Sigma-Aldrich (Missouri, USA) (CAS #: P2308-100MG)
NaAc	EMD Millipore (Massachusetts, USA) (CAS #: 127-09-3)
Sucrose	Caisson Labs (Utah, USA) (CAS #: 57-50-1)
RPMI-1640	Pan Biotech (Bavaria, Germany) (Cat. #: P04-18047)
FBS	Pan Biotech (Bavaria, Germany) (Cat. #: P30-3306)
Penicillin-Streptomycin	Pan Biotech (Bavaria, Germany) (Cat. #: P06-07100)
DMSO	NeoFroxx GmbH (Hesse, Germany) (CAS #: 67-68-5)

L-Glutamine	Pan Biotech (Bavaria, Germany) (Cat. #: P04-80100)
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3.2 METHODS

3.2.1 MicroRNA – Target Interaction Estimation for Food Allergy Project

Arachis hypogaea microRNA (miRNA) sequences were recovered from miRBase in fasta format and filtered using a custom-made script by Stuart Lucas. Additional miRNA sequences from the literature were also added manually to this fasta file. Three different tools were utilized for the miRNA: target interaction estimation (Figure 3.1). Human reference and genome annotation files GRCh38.v.104 were downloaded from ENSEMBL in both GTF and FASTA format. 3'UTR sequences were extracted from the GTF file and then converted into FASTA format using BEDtools `getfasta` command. For miRanda, the following parameters with strict alignment heuristics were chosen: energy ≤ -10 kcal/mol and score ≥ 145 . For RNAhybrid, the minimum free energy of -22 kcal/mol and 0.1 p-value cut off was applied (Zhang & Verbeek, 2010). For TargetScan, default parameters were left as they are; however, 6mer alignments were filtered out afterward. The consensus of miRNA target interactions of from all three tools were determined using R for downstream analysis.

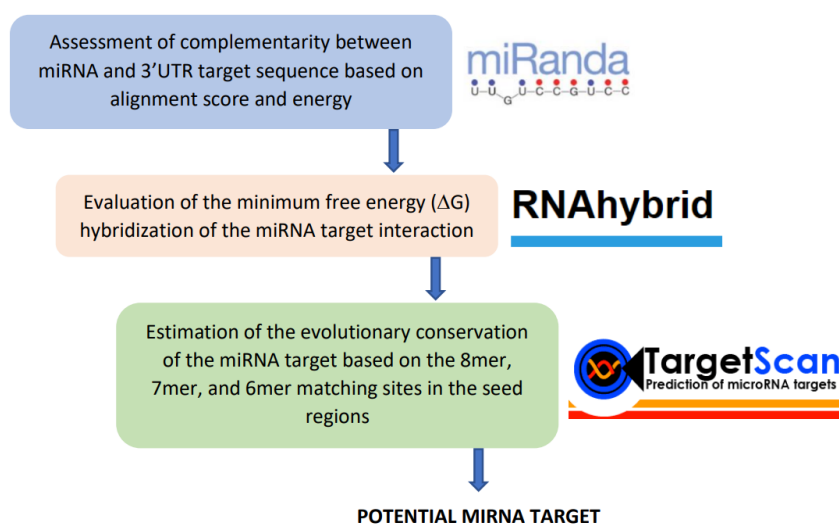


Figure 3.1: Prediction of potential food miRNA targets via machine learning algorithms.

3.2.2 RNA-seq Analysis of Peanut Small RNA Samples

Because the quantity of miRNAs in the environment is crucial for their function, peanut miRNA abundance was determined using small-RNA sequencing. 2 miRNA samples isolated directly from whole peanuts were sequenced by Illumina NovaSeq 6000 system. The quality of the raw reads was evaluated using fastqc. The reference genome for *Arachis hypogaea* was downloaded from the NCBI database in FASTA format and indexed using the `bowtie-build` command. The reads were aligned to the genome reference file using `bowtie --no-unal -f -sam`. Peanut miRNA sequences in hand were given in FASTA format to BLAST-plus to create a nucleotide BLAST database using the `makeblastdb` command. The reads were aligned with the BLAST database using `blastn -num_threads 8 -task blastn-short -outfmt 6 -max_target_seq 10 -perc_identity 0.99 -evalue 0.001`. Unique alignments were extracted from the output file in the tabular format using `sort -t$'\t' -k3 -nr`. The values were downloaded from the cluster for further processing in Excel.

To confirm the accuracy of BLAST-plus results, miRDeep2 tool was utilized in a similar manner to assess the quantity of specific miRNAs in our sequenced peanut samples. The reference genome for *Arachis hypogaea*, retrieved in FASTA format from the NCBI database, was utilized again. The miRBase was used to obtain peanut pre-miRNA hairpin and mature miRNA sequences. Because it is a member of the legume family and an evolutionary relative of peanut, mature miRNA sequences of soy bean (*Glycine max*) were also acquired from miRBase. Perl scripts of miRDeep2 package were run with the sequencing and reference files as well as related species' miRNA list; thus, known and novel peanut miRNAs were predicted. Score cut-offs between 10 and -10 were applied to reduce the number of false positives.

3.2.3 Prediction of Candidate Allergic miRNAs

The main objective at this part of the study was to determine the egg and peanut miRNAs with the highest possibility of influencing allergic reactions. For this purpose, the outcomes from miRNA-target tools were integrated with the RNA-seq result. The three miRNA-target tools were used to identify the peanut and egg miRNAs that affect the greatest number of allergy genes. The top five peanut and egg miRNAs determined using the Miranda algorithm – since it is considered to be the most specific one- were transferred into Excel files alongside

the genes they affect. These genes' functions were matched using the Reactome Pathway Database (Gillespie et al., 2021). An artificial scoring rubric was adapted to assess the miRNA functionality in a logical and comparable system. According to the scoring rubric, each signaling gene received one point, immune system genes received two points, neutrophil degranulation and Fc epsilon receptor genes received three points specifically, and the allergy hallmark genes identified through DEG analysis performed one of our former lab members received four points (İnan, 2021). The final score for each miRNA was calculated by adding together the points. To conclude, based on the RNA-seq study, the abundance of peanut miRNAs was compared.

3.2.4 Total RNA Isolation from Peanut and Egg

Different methods were used to isolate total RNA from egg samples, and their yields were compared. The Qiagen RNeasy Kit is used in accordance with the manufacturer's procedure, with the exception of a few modifications. An improved phenol-chloroform approach was employed which included several TRIzol chloroform stages, isopropanol precipitation, multiple ethanol washes, and pellet solubilization in nuclease-free water (Toni et al., 2018). In addition, hexane was included in the phenol-chloroform procedure for the total RNA isolation from egg yolk, to overcome the issues associated with high lipid content (Ferraz et al., 2004). Canik (2022) described the precise procedures used to achieve this purpose.

Nucleic acid isolation from plant tissues using cetyltrimethylammonium bromide (CTAB) buffer was adapted from the method of Doyle & Doyle (1987) for the total RNA isolation from peanut. To prevent RNA molecules from being degraded by environmental RNase enzymes, the procedure was meticulously carried out. For that purpose, peanuts were kept at -80 °C until the start of the experiment. Additionally, all the equipment and the bench surfaces were cleaned with diluted bleach, then 70% ethanol, followed by RNaseZAP. 500 mg of peanuts were pulverized on ice using mortar and pestle. Then they were thoroughly mixed with 3.75 mL 2x CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8.0). β -Mercaptoethanol at 1% concentration was also added to CTAB buffer just prior to use as a strong reducing agent. The solution was incubated at 65 °C for 30-60 minutes, and vortexed once in between. The solution was cooled to room temperature. 3.5 mL of 24:1 chloroform: isoamyl alcohol (IAA) was added to the solution and mixed by inversion. The solution was mixed slowly for 30 minutes using a rotator. The solution was centrifuged for 15

minutes at 4500 g at 4 °C. Supernatant was transferred to a new tube. Either 0.7 volume of ice-cold isopropanol or 1 volume of 4 M LiCl solution was used to precipitate RNAs in the solution. The solution was incubated at -20 °C overnight and then centrifuged at 4500 g for 10 minutes at 4 °C. The pellet was washed once with ice-cold 75% ethanol, mixed gently by inversion, and centrifuged again for 5 minutes. The supernatant was removed as much as possible and air-dried for another 5 minutes to get rid of the remaining ethanol. The pellet containing RNA was dissolved in either nuclease-free water or in a 1:1 TE: formamide solution. The sample's RNA concentration was assessed by NanoDrop, and integrity was determined by agarose gel electrophoresis.

For comparative reasons, an identical RNA isolation protocol was performed from nut buds as well. Nut buds were collected from the living plant and put in a cryogenic container with liquid nitrogen. They were also pulverized using mortar and pestle and continued with CTAB buffer incubation.

3.2.5 Peanut and Egg Nanovesicle Isolation

In order to separate nanovesicles from food samples, various procedures were used. Light absorption by nanovesicles is anticipated to start at 215 nm and continue at high levels until around 230 nm. The absorbance peak at 215 nm corresponds to the phospholipid bilayer membrane of nanovesicles. Ideally, their absorbance should then decrease, providing absorbance values that are nearly equal to zero around 340 nm. Using a NanoDrop instrument to measure optical densities of final samples at crucial points, the efficacy of our methods was assessed (Table 3.3).

Table 3.3: Nanovesicle isolation methods.

Method	Isolation Principle	Success
Ultracentrifugation & Ultrafiltration	size and density	positive
Ultracentrifugation & PEG purification	size and chemical precipitation	negative
Sucrose Cushion & TCA Purification	flotation densities	positive

Ultracentrifugation and Ultrafiltration

This protocol was adapted from the method of Aquilano *et al.* (2019) (Figure 3.2). Peanuts and eggs were kept at 4 °C until the start of the experiment. Peanuts were pulverized using mortar and pestle. The albumen and yolk of two eggs were combined and homogenized for raw egg samples. To obtain cooked egg samples, heat was applied for 15 minutes to the mixed albumen and yolks of two eggs using a water bath. 3.5 grams of each sample were suspended in PBS with the 1:10 w/v ratio and mixed well. Samples were centrifuged for 15 minutes at 20,000 g at 4 °C, and the infranatant was collected. Samples were centrifuged at 10,000 g for 1 hour at 4 °C and the supernatants were collected. They were passed through 0.45-micron filters and then centrifuged again at 16,500 g for 1 hour at 4 °C. The supernatants were collected and centrifuged at the maximum speed, 100,605 g, for 70 minutes at 4 °C. Supernatants were discarded, and the pellets were dissolved in 500 µl PBS. Solutions were filtered using 0.45-micron filters and centrifuged at 16,500 g for 10 min at 4 °C. Supernatants that contain nanovesicles were transferred into new Eppendorf tubes and stored at -80 °C for further processing.

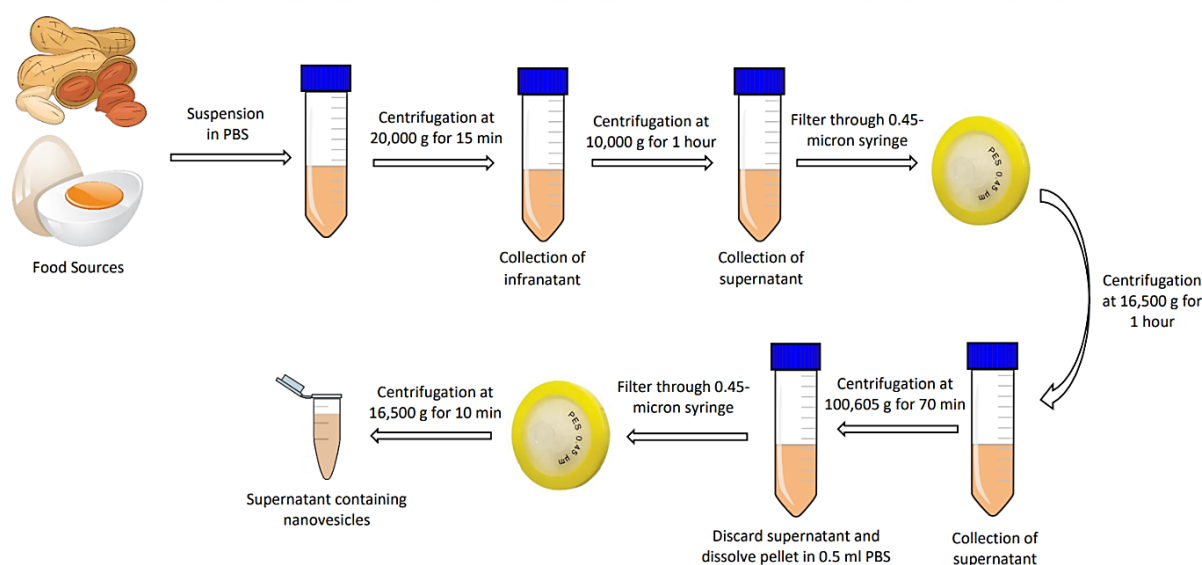


Figure 3.2: Nanovesicle isolation from food sources protocol using ultracentrifugation and ultrafiltration (Aquilano *et al.*, 2019).

Peanut Nanovesicle Isolation with Ultracentrifugation and PEG

This protocol was adapted from Rider *et al.* (2016). Peanuts were ground with a household-type blender and stored at 4 °C overnight. 20 g peanut was mixed with 200 ml PBS (1:10 w/v) in 250 ml centrifuge tubes. Samples were centrifuged at 20,000 g for 15 minutes at 4 °C and infranant was collected. Samples were centrifuged at 10,000 g for 1 hour at 4 °C and supernatant was collected. 1:1 v/v 2x 24% PEG solution (24% PEG 6000, 1 M NaCl) was added to samples and mixed by inversion. Samples were incubated for approximately 24 hours at 4 °C and then centrifuged at 16,500 g for 1 hour at 4 °C. The supernatant was discarded and the pellets were dissolved in 750 µl PBS. Afterward, samples were divided into three even parts to apply three different purification protocols. The first part of the samples were filtered using a 0.45-micron filter and then centrifuged at 16,6 g for 10 minutes at 4 °C. The supernatant was discarded and the pellet was dissolved in 500 µl PBS. For the second part of the samples, PEG purification was applied. Samples were diluted in 5 ml PBS and then an equal volume of 2x 10% PEG solution (10% PEG 6000, 1 M NaCl) was added to adjust the final concentration of PEG to 5%. The solution was incubated at 4°C for 4 days and then centrifuged at 16,500 g for 1 hour at 4 °C. The supernatants were discarded and the pellets were dissolved in 750 µl PBS. Samples were filtered using 0.45-micron filters and centrifuged again at 16,600 g for 10 minutes at 4 °C. The supernatants were discarded and pellets were dissolved in 500 µl PBS. For the third part of the samples, ultracentrifugation purification was applied. The sample volumes were completed to 30 ml by the addition of PBS. The samples were centrifuged at 100,000 g for 70 min at 4 °C. The pellet was dissolved in 750 µl PBS, passed through a 0.45-micron filter, and centrifuged at 16,600 g for 10 min at 4 °C. The pellet was dissolved in 500 µl PBS.

3.2.6 Proteinase K Treatment of Egg Nanovesicle Samples and SDS-PAGE

Proteinase K (20 mg/ml) was used to treat egg samples that were thought to be contaminated with protein. 1 µl of Proteinase K was added to each 100 µl of the nanovesicle sample and thoroughly mixed by pipetting. Solutions were incubated for 30 minutes at 38 °C using a thermostat. Tubes were placed in a freezer to stop the reaction and to be stored until further processing. A precast SDS gel was used to separate and examine the proteins in these

samples. 20 μ l sample was combined with 10 μ l of a 3x protein loading dye and heated using a thermostat at 95 °C for 5 minutes. Samples were loaded to gel and run at 200 V for 10 minutes. Gel was imaged after being photoactivated by UV irradiation for 2.5 minutes using the BIO-RAD Gel Doc EZ Imager.

3.2.7 Purification of Peanut and Egg Nanovesicle Samples Using Sucrose Cushion

Aquialano *et al.*'s approach was used to isolate peanut and egg nanovesicles from tissue homogenates; nevertheless, contamination was identified based on the unexpected peaks in the spectrometric analysis. A sucrose cushion procedure was used to purify the samples (Figure 3.3) (Van de Vlekkert *et al.*, 2020). The sucrose gradient solution containing 10 mM Tris-HCl, pH 7.4/2 mM NaAc was prepared. Sucrose gradient working solutions (SGWS) were adjusted in accordance with Table 3.4. 500 μ l of NV samples were mixed with 1.5 ml of 0.25 M SGWS. Into an ultracentrifuge tube, 3 ml 2.0 M, 5 ml 1.3 M, 5 ml 1.16 M, 4 ml 0.8 M, and 4 ml 0.5 M sucrose solutions were transferred gently from bottom to top respectively. By addition of the 0.25 M sucrose solution containing nanovesicles on top, gradient was completed. The gradient was centrifuged at 100,000 g for 2.5 hours using a fixed-angle rotor. The gradient was divided into 5 fractions of 4.6 ml each. 1.1 volume of 100% TCA was added to each fraction and incubated at room temperature for 10 minutes. Each fraction was centrifuged at 10,000 g for 10 min at room temperature and the pellets were dissolved in ice-cold PBS.

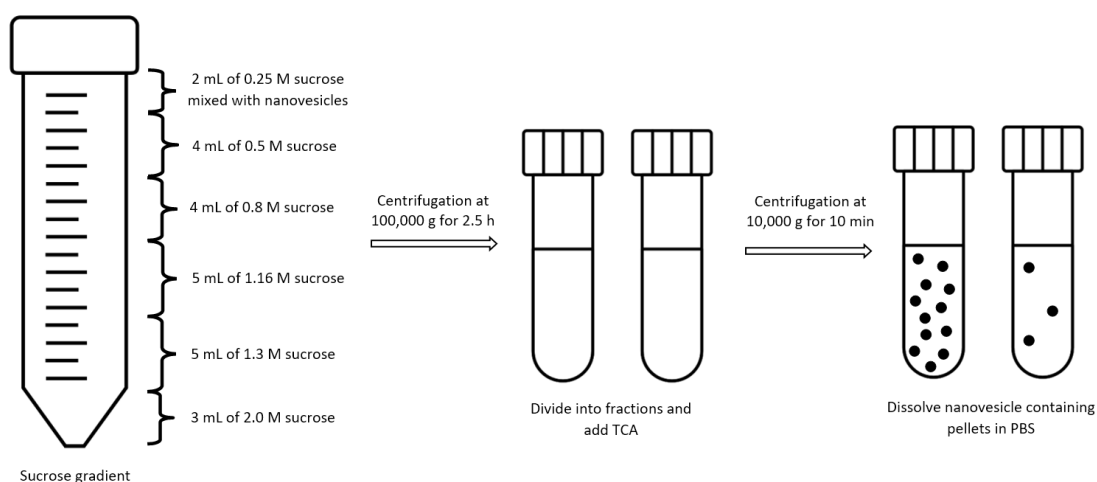


Figure 3.3: Sucrose cushion protocol for nanovesicle purification (Van de Vlekkert *et al.*, 2020).

Table 3.4: Sucrose gradient working solution preparation.

Final Molarity	Sucrose (g)	SGWS volume (ml)
2.0	2.74	4.0
1.3	2.67	6.0
1.16	2.38	6.0
0.8	1.37	5.0
0.5	0.86	5.0
0.25	0.26	3.0

3.2.8 Imaging of Nanovesicle Samples Using Scanning Electron Microscopy (SEM)

Pure nanovesicle samples with high concentration were chosen from all samples in order to obtain clear SEM images. Due to the high density of nanovesicles and salts coming from PBS, samples were diluted 1:3 in nuclease-free water. 4 μ l of diluted samples was transferred on a glass slide and air-dried overnight at room temperature. The photos were captured the day after the sample preparation using a Zeiss Supra 35VP field emission scanning electron microscope. The Extra High Tension (EHT) was set to 3.00 kV, and the Working Distance (WD) was set at 8.8 mm.

3.2.9 Nanovesicle Characterization with Nanovesicle Tracking Analysis (NTA)

The Malvern Panalytical NanoSight NS300 instrument was used to carry out Nanovesicle Tracking Analysis (NTA) to measure the concentration and size distribution of the nanovesicle samples. Nanovesicle samples were diluted 1:1000 with PBS. The instrument records three movies that each last for 60 seconds and then allows users to evaluate their samples using the so-called "particle-by-particle methodology" based on the Brownian movements of individual particles. The threshold value was established based on how distinct and visible individual nanovesicles were in the movies prior to analysis. Three analyses were conducted and averaged.

3.2.10 Cultivation of KU812 Cell Line for Functional Analysis

The KU812 cell line is characteristic of basophils, a kind of human white blood cell. They contribute to allergic reactions by degranulating and secreting histamine, heparin, tryptase, and cytokines. According to the manufacturer's product sheet (ATCC CRL-2099, 2023), RPMI-1640 cell media enhanced with 10% FBS and 1% Penicillin-Streptomycin was used to cultivate the KU812 cell line. It is a suspended cell line that requires 95% air and 5% CO₂ incubation conditions at 37 °C. KU812 cells were seeded at a density of 1.0×10^5 cells/ml in a T75 cell culture flask in 10 mL complete RPMI-1640 medium. Once a week, the cells were centrifuged at 300 g for 5 minutes at 4 °C and transferred to a new flask with fresh medium. Every other day, β -mercaptoethanol was added at 0.05 mM final concentration to clean the medium from wastes and ameliorate cell viability. When the components of the RPMI-1640 medium were depleted or deteriorated, L-Glu (1%) was added to the medium. For cryopreservation, freeze medium containing 5% DMSO in complete RPMI-1640 cell medium was used. To slow the cell freezing process for healthy long-term storage, Thermo Scientific™ Mr. Frosty™ Freezing Container was utilized (Thermo Scientific, 2010).

3.2.11 Flow Cytometry

To obtain accurate flow cytometry findings, the viability of KU812 cells was set to at least 70%. Cells were centrifuged at 300 g for 5 minutes at 4 °C and dissolved in the flow cytometry staining buffer (FACS) (1x PBS, 5% FBS, 0.5 mM EDTA, filtered through 0.2 μ m filter). Cells were counted using a trypan blue solution with a hemocytometer. For each sample type, $\sim 3 \times 10^5$ cells were dissolved in 300 μ l FACS buffer and transferred into a 96-well plate. For the calcium ionophore (CI) treated group, 1 mM CI diluted in DMSO was added and mixed by pipetting. For the control group, nothing was added. The cells were incubated for 1 hour at 37 °C. Then the cells were collected and transferred into Eppendorf tubes. Cells were centrifuged at 300 g for 5 minutes at 4 °C and washed once with 150 μ l FACS buffer. The supernatant was removed, and the cells were dissolved in 200 μ l FACS buffer. FITC-conjugated CD63 and/or PE-conjugated CD203c antibodies were diluted 1:100 in FACS buffer. 10 μ l of the antibody solution was added to the cells and incubated for 30 min at RT in the dark. The cells were centrifuged again and washed once with FACS buffer. The cell pellet was dissolved in 100 μ l FACS buffer finally. Before measurement, another

200 μ l of FACS buffer was added and mixed thoroughly by pipetting to prevent cell clustering. Fluorescence measurements were performed using BD LSRFortessa™ Cell Analyzer.

In the nanovesicle transfection experiments, nanovesicle samples were diluted with PBS and 10 μ l of nanovesicle solution were incubated with $\sim 3 \times 10^5$ cells for 1 hour at 37 °C in 96 well plate. The cells were then collected, centrifuged, and washed once with FACS buffer. The cell pellet was dissolved in 200 μ l of FACS buffer and antibody staining protocol was applied as previously stated.



4 RESULTS

4.1 miRNA-Target Prediction

One of our former lab members (İnan, 2021) identified 1054 differentially expressed genes in the T cells of the food allergy patients (Martino et al., 2016). To investigate where peanut miRNAs may interact with these human DEG genes, three different miRNA-target tools are utilized. According to the agreement of these tools, *Arachis hypogaea* miRNAs have the potential to modify 14 422 human genes, 711 of which are the DEG genes for food allergy (Figure 4.1). This figure implies that peanut miRNAs may regulate over 65% of these food allergy related genes.

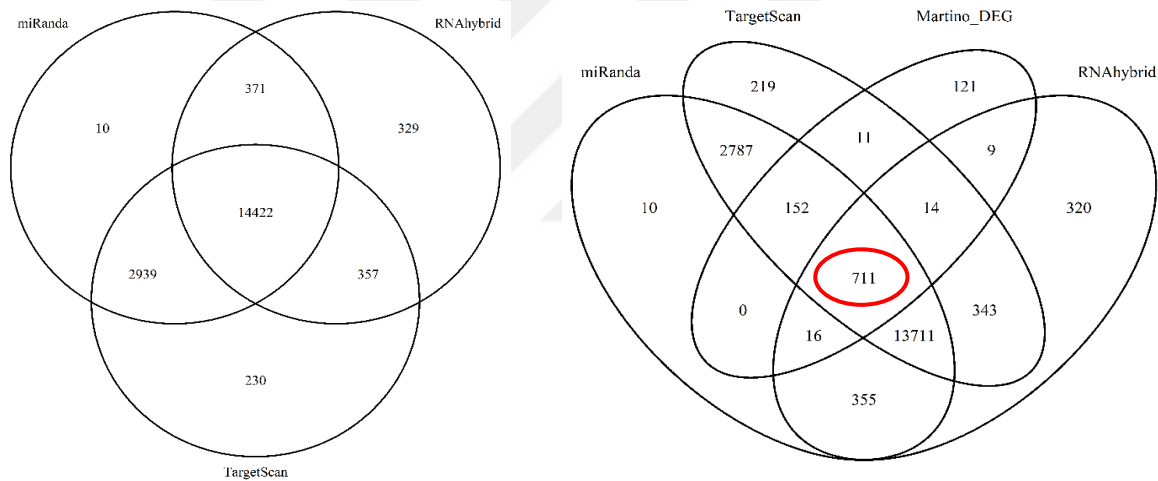


Figure 4.1: Venn diagram of peanut miRNA: target prediction tools on the left, and allergic DEGs, on the right.

4.2 Small RNA-seq Analysis

The abundance of miRNAs in the environment is critical for their functionality; therefore, we used small-RNA sequencing to quantify the number of miRNAs present in peanuts. One of my former colleagues collected miRNA directly from peanut samples using SIGMA mirPremier microRNA isolation kit (Canik, 2022), and two of these high-quality samples were sequenced with Illumina. The table 4.1 summarizes the alignment results. The sample

collection, sequencing, filtering, and alignment methods were all effective, as evidenced by the high number of clean reads even after filtering and Bowtie's excellent alignment percentages.

Table 4.1: Small RNA-seq analysis results.

	Number of Reads	Number of Clean Reads	Number of Reads with at least one Reported Alignment
Sample 1	10 497 461	10 177 839	9 443 719 (92.79%)
Sample 2	9 929 493	9 474 616	9 161 541 (96.70%)

Peanut miRNAs and sequences are assembled in a FASTA file based on literature review and the miRBase data bank. The most common peanut miRNAs were found using this peanut miRNA list and command-line BLAST+ program alignment (Figure 4.2, Appendix Table 7.4). As expected, the results of the two samples agree with one another. This alignment has determined miR6478, miR159a, and miR319 as the three most abundant miRNAs in peanuts.

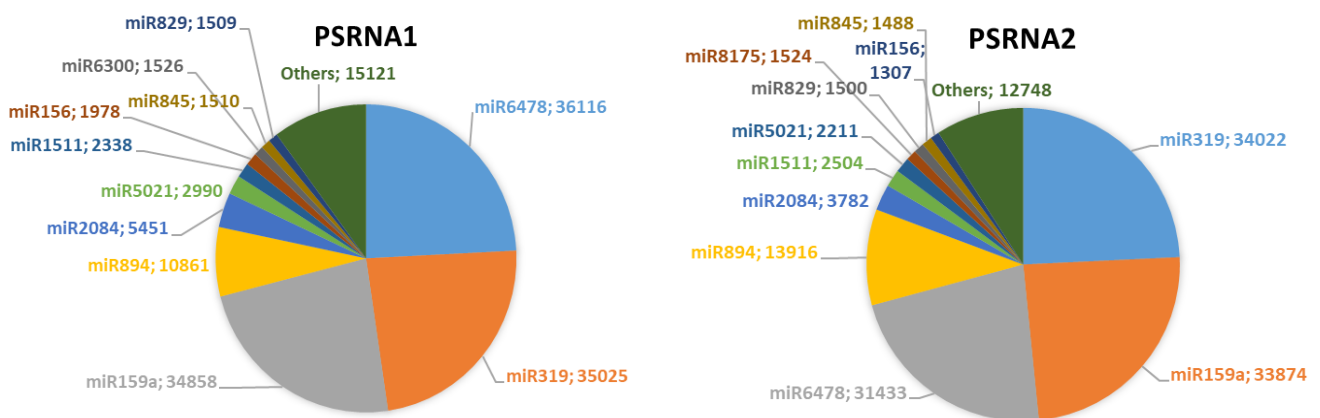


Figure 4.2: Most abundant peanut miRNAs according to small RNA-seq.

To quantify the miRNAs present in a sample, miRDeep2 algorithm takes into account the process by which the Dicer protein cleaves the pre-miRNA loop. This process yields three products of varying lengths: a loop structure, a star strand, and mature miRNA. The loop

structure is rapidly degraded, whereas the star strand is discarded later when helicases unwound it from the mature miRNA strand (Friedländer et al., 2012). By employing a unique method considering aforementioned miRNA biogenesis, miRDeep2 package discovers known and novel miRNAs in a sequenced sample. Because BLAST alignment is a fairly basic one utilized for generic use and miRNA biogenesis is complex with several processing steps, we decided to use miRDeep2 to carry out a more tailored analysis for our study. Score cut-offs were limited to between 10 and -10 to eliminate false positives. As the miRDeep2 score increases, the number of estimated false positives are reduced; correspondingly, the miRNAs are being ranked in the results table according to their accuracy rather than their total read counts. Interestingly, miRDeep2 has found only 4 miRNAs in our samples as opposed to BLAST-plus which has counted more than 65 miRNAs (Table 4.2). Nonetheless, miRDeep2 acknowledges that many more miRNAs could be discovered in the sample using a simple alignment methodology (Table 4.3). Because the miRDeep2 algorithm searches for both 5p and 3p mature miRNA products and identifies the miRNA only if both are present, 5p and 3p readings are counted separately in the table. Another key result from miRDeep2 is the long list of novel peanut miRNAs (Appendix Table 7.2). 215 novel peanut miRNAs were estimated to be present in our sample, with some having as many as 19 400 read counts. Friedländer et al. (2012) claimed that the accuracy rate of finding novel miRNAs is over 99% in their miRDeep2 article. Based on these findings, we can conclude that our understanding of peanut miRNAs is significantly limited, and there are many more miRNAs awaiting discovery.

Table 4.2: Mature miRBase miRNAs detected by miRDeep2. gma is the symbol for the soy bean, precursor coordinate indicates the genomic location of the original miRNA according to the reference peanut genome.

mature miRBase miRNA	Total Read Count	miRDeep2 Score	Estimated Probability that the miRNA is a True Positive	Example miRBase miRNA with the same Seed	Precursor Coordinate
ahy-miR167-5p	497	2.5×10^2	0.59 ± 0.09	gma-miR167g	NC_037636.1:157637805..157637883:+
ahy-miR160-5p	244	1.2×10^2	0.59 ± 0.09	-	NC_037621.1:6777537..6777623:+

ahy-miR3518	437	-17	0.00 ± 0.00	-	NC_037626.1:1788030..1788081:+
ahy-miR3514-3p	1288	-18	0.00 ± 0.00	-	NC_037637.1:10376044..10376105:+

Table 4.3: Mature miRBase miRNAs not detected by miRDeep2; 5p and 3p read counts are detected in the miRNA hairpin structure.

miRBase Precursor ID	Total Read Count	5p read counts	3p read counts	Remaining Reads
ahy-MIR159	63792	0	63770	22
ahy-MIR156c	6683	6683	0	0
ahy-MIR3516	1013	999	0	14
ahy-MIR3512	218	0	218	0
ahy-MIR3511	158	121	11	26
ahy-MIR3509	625	14	120	491
ahy-MIR408	29	0	29	0
ahy-MIR394	17	17	0	0
ahy-MIR398	18	18	0	6
ahy-MIR3510	15	13	0	2
ahy-MIR156b	8	0	16	2
ahy-MIR3517	13	3	0	10
ahy-MIR3515	5	0	0	5

4.3 Prediction of Candidate Dietary Allergic miRNAs

The peanut and egg miRNAs regulating the food allergy genes were determined by miRNA-target algorithms (Figure 3.1). It is critical to identify dietary miRNAs that are most likely to cause food allergies so that their role can be validated in wet lab. Because the Fc epsilon

receptor and neutrophil degranulation are two of the most significant allergy pathways, a miRNA that particularly regulates genes in these pathways would be more allergic. Furthermore, miRNAs targeting the hallmark allergy genes determined by İnan (2021) have the potential to trigger food allergies. Considering these factors, artificial scores are assigned to the miRNAs with the greatest number of targets (Table 4.4 & 4.5). Based on the findings, these miRNAs are strong candidates for food allergies, and they could be targeted for further research.

Table 4.4: Predicted allergic peanut miRNAs.

	miRNA-Target Total Number	Signalization Pathway (x1)	Immune System Pathway (x2)	Allergic Reaction Pathway (x3)	DEG Pathway (x4)	Final Score
ahy-miR164	715	8	7	3	0	31
ahy-miR169b	643	6	3	1	0	15
ahy-miR159a	642	7	5	1	0	20
ahy-miR399	634	6	5	2	0	22
ahy-miR482d-3p	622	16	11	2	1	48

Table 4.5: Predicted allergic egg miRNAs.

	miRNA-Target Total Number	Signalization Pathway (x1)	Immune System Pathway (x2)	Allergic Reaction Pathway (x3)	DEG Pathway (x4)	Final Score
gga-miR-1587	2333	400	219	86	0	1096
gga-miR-2127	2296	400	239	88	0	1142
gga-miR-1635	1924	303	186	82	0	921
gga-miR-6593-5p	1630	253	155	60	0	743
gga-miR-1594	1512	284	163	58	0	784

4.4 Total RNA Isolation from Peanut and Egg

Total RNA isolation from peanut and egg was performed to confirm the existence of miRNAs by downstream applications such as reverse transcription polymerase chain reaction (RT-PCR) and RNA-seq analysis. To achieve this goal, total RNA was isolated using a CTAB buffer technique for peanuts; however, it was observed that the integrity of samples was consistently impaired. Employment of strict sterilization methods to protect the RNA integrity was inadequate. To pinpoint the source of the problem, total RNA isolation procedure was performed concurrently from the buds of a nut plant and from peanuts. In comparison to RNA isolation from nut buds, high quantities and good purity of peanut RNA could be obtained (Table 4.6). However, the agarose gel results demonstrate that the peanut RNA is not intact (Figure 4.3). Ribosomal RNA bands from nut buds are clearly visible despite their lower RNA concentration. In the light of these findings, it was concluded that our RNA isolation technique was a success. Nonetheless, it also proved unlikely to isolate intact total RNA from roasted peanut samples purchased from local markets. Picking peanuts directly from a live plant, if it is possible, may be an option to achieve the desired outcomes in this experiment.

Several methods were used to isolate total RNA from eggs, including an enhanced phenol-chloroform approach, the Qiagen RNeasy Kit, and the introduction of hexane steps. Canik (2022) has previously published the results.

Table 4.6: Peanut total RNA isolation results.

Sample	RNA Concentration (ng/ μ l)	A_{260}/A_{280}	A_{260}/A_{230}
Peanut Total RNA	711.2	1.02	2.42
Nut Bud Total RNA	42.7	0.701	3.87

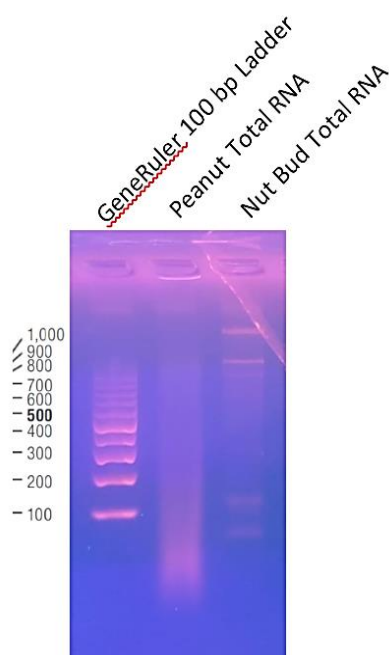


Figure 4.3: Peanut and nut bud total RNA isolation agarose gel image.

4.5 Nanovesicle Isolation with Ultracentrifugation and Ultrafiltration

Dietary nanovesicles and their content is another focus of our study. To characterize and prepare pure nanovesicles from our food sources, several isolation protocols were attempted (Table 3.3). It was important to collect high-quality nanovesicle samples since they would be used in functionality assays on our basophil cell line.

Nanovesicle samples should absorb most at 215 nm, continue to absorb strongly at 230 nm, and then begin to absorb less significantly at about 340 nm. The degrees of absorption of isolated nanovesicles are determined by UV-visible spectrophotometry (Table 4.7). Nanovesicles are highly concentrated and pure in the albumen and peanut samples. High concentrations of nanovesicles were found in the yolk and cooked egg - 2 samples; however, some impurities may have remained in the solution judging by the small peaks observed at around 340 nm. Cooked egg nanovesicle -1 sample has a very high degree of contamination and needs extra purification procedures.

Table 4.7: Peanut and egg nanovesicle isolation using ultracentrifugation and ultrafiltration results.

Sample	215 nm	230 nm	340 nm
Peanut Nanovesicle	0.674	0.325	0.068
Albumen Nanovesicle	0.630	1.670	0.001
Yolk Nanovesicle	0.684	1.987	0.355
Cooked Egg Nanovesicle - 1	0.556	0.678	1.576
Cooked Egg Nanovesicle - 2	0.998	1.540	0.132

4.6 Proteinase K Treatment with Egg Nanovesicle Samples and SDS-PAGE

Impurities such as proteins that have accumulated around the nanovesicles due to surface contacts or impurities that could not be removed using the ultracentrifugation and ultrafiltration protocols are believed to be present in nanovesicle samples with high absorbances at 340 nm. It is crucial to detect the exact source of contamination and establish a strategy for the purification of the nanovesicles. Proteinase K is an enzyme that hydrolyzes peptide bonds, thus degrading the proteins (Ebeling et al., 1974). To a part of the samples, proteinase K was applied to decide whether the contaminant is a protein. Proteinase K treated samples were analyzed using an SDS-PAGE (Appendix Figure 7.1). According to the SDS-PAGE data, proteinase K treatment was effective in eliminating the contaminants. This suggests that the contaminants were egg proteins, which were also approved by literature review (Appendix Table 7.3).

4.7 Peanut Nanovesicle Isolation with Ultracentrifugation and PEG

As an alternative to the ultracentrifugation and ultrafiltration protocol, a procedure that combines ultracentrifugation with PEG purification was used to isolate nanovesicles from peanuts. However, the protocol was ineffective as can be concluded from the very low final sample concentration (Table 4.8).

Table 4.8: Peanut nanovesicle isolation using ultracentrifugation and PEG results.

Sample	215 nm	230 nm	340 nm
Peanut Nanovesicle	0.053	0.024	0.005

4.8 Nanovesicle Purification with Sucrose Cushion Protocol

Sucrose cushion protocol was applied for purification to the contaminated nanovesicle samples isolated with ultracentrifugation and ultrafiltration. For this purpose, one albumen and one cooked egg sample with high absorbances at 340 nm were chosen. After ultracentrifugation with a sucrose cushioning, samples were separated into 5 fractions, and nanovesicles within these fractions were precipitated using TCA. Spectrophotometry is used to quantify the amounts and purities of nanovesicles in each fraction initially (Table 4.9 & 4.10). The sucrose cushion procedure combined with TCA precipitation successfully removes contaminants from nanovesicle samples, according to UV-visible spectrum analysis. In the cooked egg fraction 1, this method concentrated the nanovesicles based on the absorbance at 215 nm, while removing the purities as can be deduced from the decreased 340 nm absorbance. This demonstrates the sucrose cushion protocol's outstanding performance in purifying the egg nanovesicle samples. While the difference between the original sample and the most successful fraction is less obvious in albumen, the peaks around 340 nm in the original samples have been removed in each fraction (Figure 4.4).

Table 4.9: Cooked egg nanovesicle purification using sucrose cushion protocol results.

Sample	215 nm	230 nm	340 nm
Original Cooked Egg NV	0.777	2.226	0.354
Cooked Egg Fraction 1	1.287	0.563	0.164
Cooked Egg Fraction 2	0.669	0.170	0.031
Cooked Egg Fraction 3	0.519	0.132	0.017
Cooked Egg Fraction 4	0.230	0.053	0.007
Cooked Egg Fraction 5	0.476	0.158	0.054

Table 4.10: Albumen nanovesicle purification using sucrose cushion protocol results.

Sample	215 nm	230 nm	340 nm
Original Albumen NV	0.578	1.687	0.015
Albumen Fraction 1	0.287	0.085	0.028
Albumen Fraction 2	0.349	0.066	0.005
Albumen Fraction 3	0.274	0.060	0.011
Albumen Fraction 4	0.491	0.106	0.011
Albumen Fraction 5	0.293	0.068	0.006

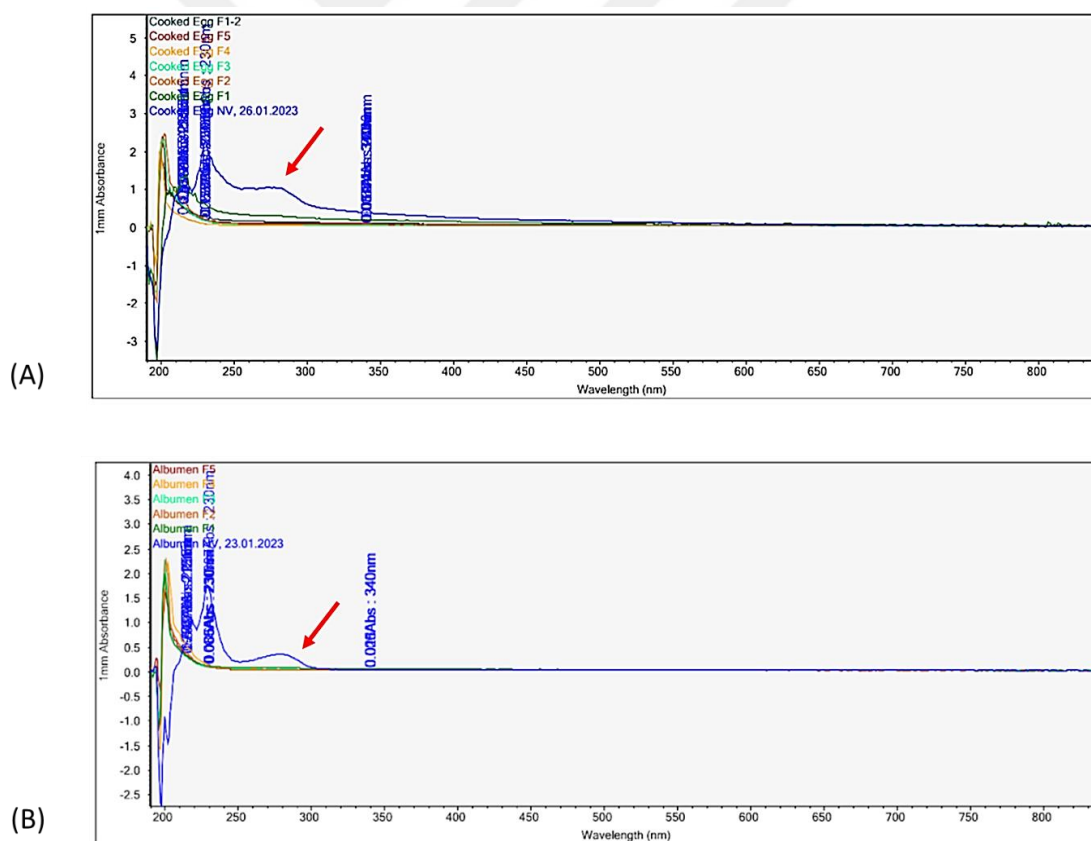


Figure 4.4: Nanovesicle absorbance values of sucrose cushion protocol, (A) cooked egg, (B) albumen. Red arrows show the peaks in the original samples caused by contaminants. The sucrose cushion procedure successfully eliminates these peaks, as can be seen.

4.9 SEM Imaging of Peanut and Egg Nanovesicle Samples

SEM instruments enable users to obtain high-resolution, three-dimensional images of even extremely small particles at nano sizes, which is valuable in topographical, analytical, and morphological analysis (Choudhary & Priyanka, 2017). Visual inspection of nanovesicles using conventional light microscopes is challenging due to their small size; hence, SEM is widely utilized to characterize nanovesicles.

SEM was used in our study to examine nanovesicle samples isolated from food sources (Figure 4.4). Red arrows are used to point at particles that we believe are nanovesicles. The edges appear to be brighter than the middle of the circle, which appears to be darker. That happens as a result of the density and spherical form of the nanovesicles.

The isotonic 1x PBS solution, which contains 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , was used to dissolve and preserve the isolated nanovesicles from the food samples. After air-drying, a crystalline appearance might be seen due to the salt content in the PBS, as it is visible in the bottom right corner of the (d) (Figure 4.5). This crystalline look was reduced after dilution with nuclease-free water so a clearer view of the nanovesicles could be obtained.

Peanut nanovesicles on (a) are larger, denser, and uniformly round in shape. However, the egg nanovesicle samples (b, c, and d) are less dense and smaller in size. The background of cooked egg nanovesicles (d) is unclean and hazy. This is because nanovesicle isolation from the cooked egg was performed earlier in contrast to other groups. The stability of these nanovesicles may have been impacted through undergoing numerous freeze-thaw cycles despite the sample being stored at $-80\text{ }^{\circ}\text{C}$.

Using ImageJ software version 1.54, the average diameters of peanut, albumen, yolk, and cooked egg nanovesicle samples are measured as 167.2, 112, 110.8, and 136.5 nm, respectively.

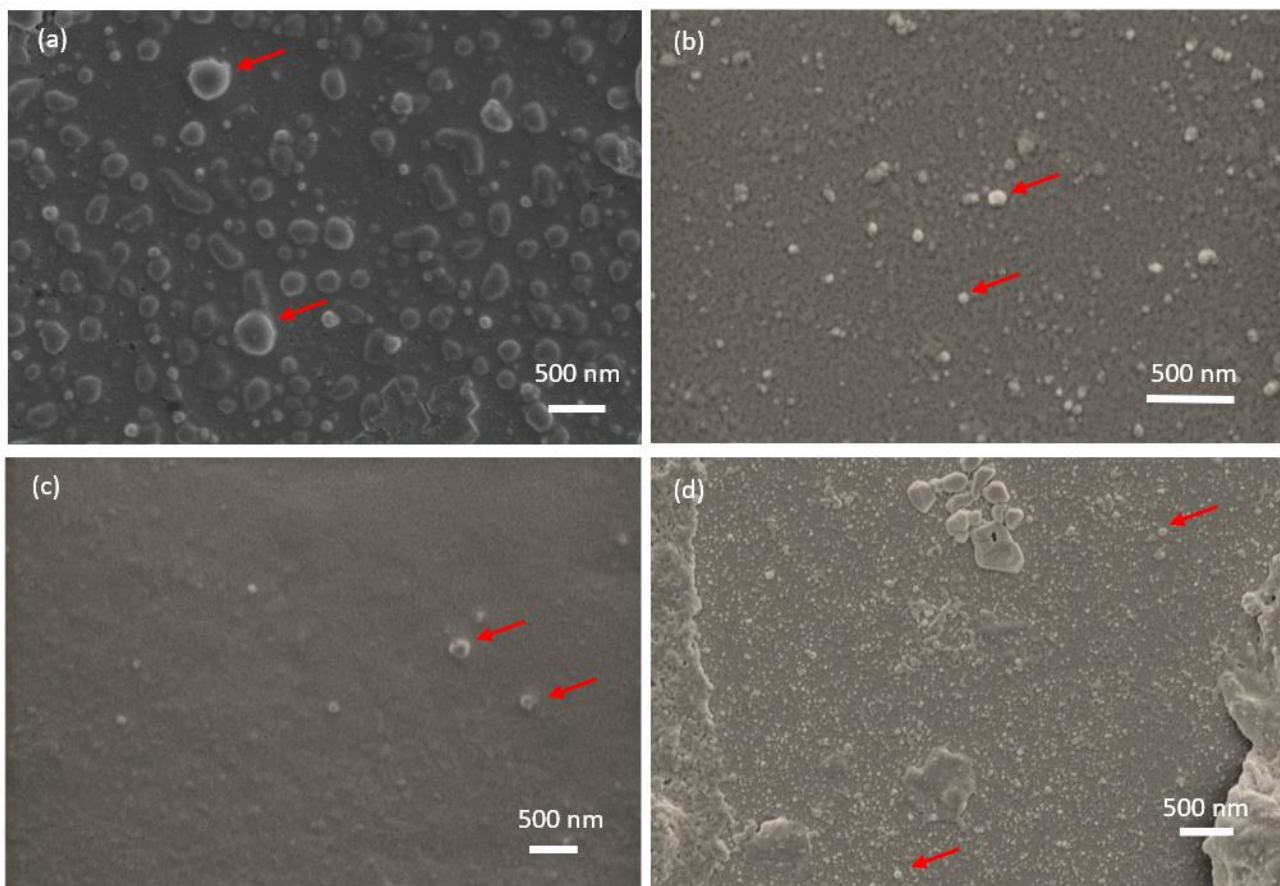


Figure 4.5: SEM images of (a) peanut, (b) albumen, (c) yolk, and (d) cooked egg nanovesicles.

4.10 Nanovesicle Tracking Analysis (NTA)

Nanovesicle tracking analysis is recommended by many researchers in the extracellular vesicle field, since it yields accurate and reproducible results regarding the number of particles and their size distribution (Comfort et al., 2021). Accordingly, we utilized NTA analysis to quantify our cooked egg nanovesicles and evaluate their size distribution. Nuclease-free water was used to dilute the samples so they would seem clearer in the movies, and analysis was carried out using Malvern Panalytical NanoSight NS300 Instrument. This device, like its counterparts, concentrates laser beams on the nanovesicles within liquid and records movies when the nanovesicles scatter light. The NTA software calculates flow rate as a function of size by tracking each nanovesicle's Brownian motion (Comfort et al., 2021).

Multiple cooked egg nanovesicles can be seen in the screenshot from one of the movies as white spheres on a black background (Figure 4.6). According to the size distribution graph (Figure 4.7), the majority of egg nanovesicles are between 150 and 200 nm in size, with only

a few reaching 683 nm in size. The concentration of this sample measured as 1.7×10^{11} particles/mL, which is compatible with its high absorbance peak around 215 nm, with 0.556 absorbance value.

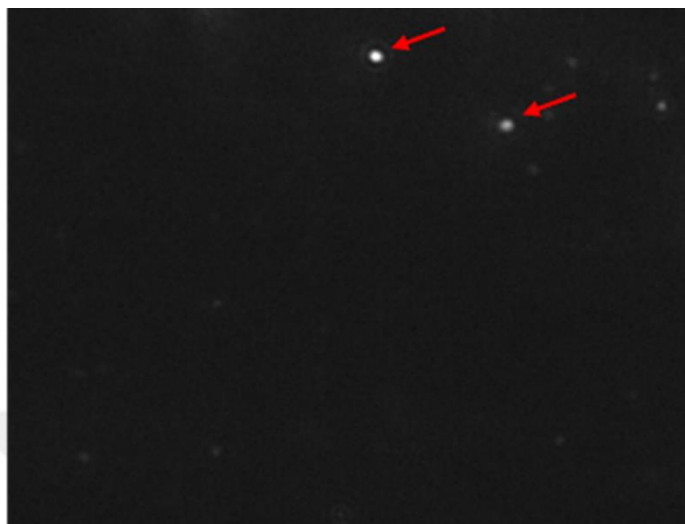


Figure 4.6: Screenshot from NTA movie of cooked egg nanovesicle sample.

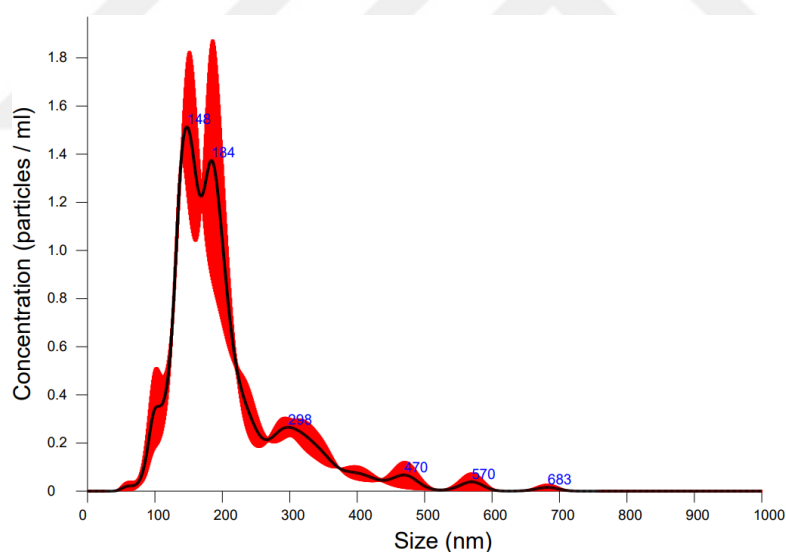


Figure 4.7: Size distribution of cooked egg nanovesicle samples.

4.11 Cultivation of KU812 Cell Line for Functional Analysis

KU812 basophilic cell line was used to conduct functional assays with dietary nanovesicles. Basophils play an important part in food allergies because they induce immune reactions when they recognize the allergen. Degranulation is the process by which particular

communication molecules such as histamine and tryptase are secreted into the extracellular environment by basophils, in order to initiate the allergic responses. Basophils undergo physiological changes as a result of this process, and certain substances can be detected in the cell media.

KU812 cells were cultivated in RPMI-1640 cell medium enriched with FBS and Penicillin-Streptomycin in accordance with the product sheet provided by the manufacturer (ATCC, 2023). Physical conditions of cells are checked daily with Zeiss inverted light microscope (Figure 4.8). Additionally, total cell number, live cell number and cell viability are assessed using a hemacytometer after trypan blue staining.

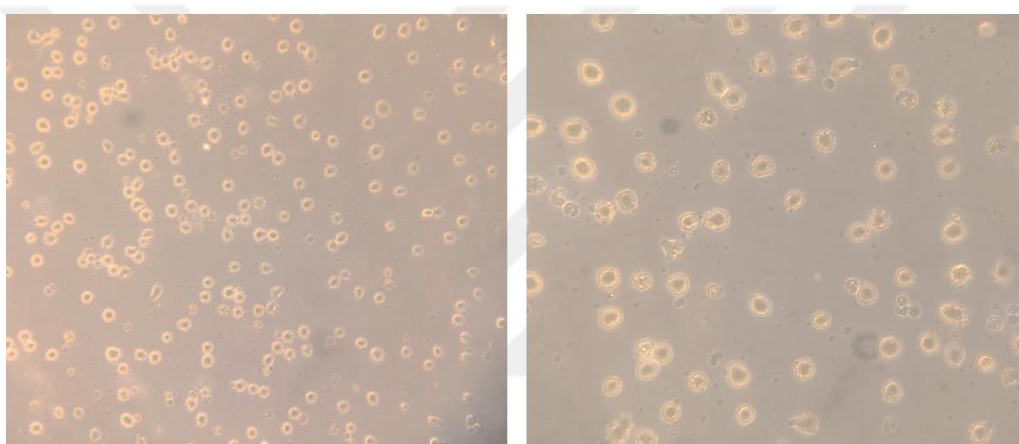


Figure 4.8: KU812 cell line after 5 days of culturing; 20x on the left, 40x on the right.

4.12 Flow Cytometry

Basophil activation test (BAT) a flow cytometric assay to quantify the degranulation of the basophil cells when an allergen is introduced. For that purpose, CD63 and CD203c antibodies are employed as activation markers (Figure 4.9). Upon exposure of allergen, CD203c and CD63 expression on the cell membrane is rapidly upregulated, allowing researchers to describe the degranulation (Briceno Noriega et al., 2021). Flow cytometry software generates numerical values for each sample, which are referred to as mean fluorescence intensity (MFI). The stimulation index, which is the ratio of the MFI of the stimulated sample to that of the negative control, is used to compare the effects of treatments.

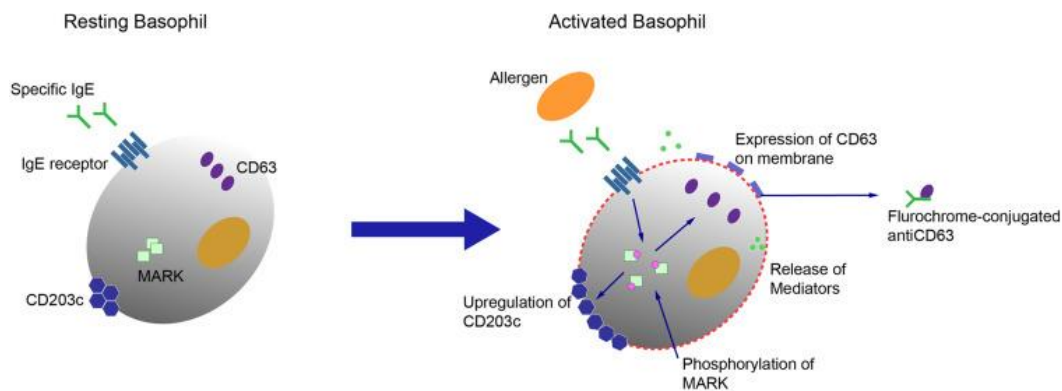


Figure 4.9: Basophil activation test working principle (Briceno Noriega et al., 2021). CD63 and CD203c are the most common markers that are upregulated when basophils are activated.

Flow cytometric measurements were performed step-by-step to make sure that the optimal experimental conditions are met. The efficiency of the CD63 antibody staining is determined by a dilution series of FITC-conjugated CD63 antibody. Non-stained cells are compared with 5, 10 and 20 μ l 1:100 FACS buffer diluted CD63 antibody (Figure 4.10). The results of the 20 μ l antibody treatment sample were discarded because the cell pellet was lost during the last washing step and the flow cytometry measurements were not convincing. The increase in the MFI indicates that the staining was successful and adequate even at the lowest antibody concentration. After this pilot experiment, threshold values of parameters were adjusted so that the peaks observed on the left side of the plots were eliminated to obtain clearer data.

Activation of basophil cells with CI treatment is tried with different concentrations and incubation durations. 100 and 200 nM final CI concentrations with 1-hour incubation gave significant increase in the MFI, showing the efficiency of basophil activation by this chemical (Figure 4.11). During the analysis, doublet cells are identified and removed using a gate based on their distance from the linear regression line in the FSC-A vs FSC-H scatter plot. Similarly, in the FSC-A vs SSC-A scatter plot, non-cell particles such as cell debris are filtered out based on their segregation from the main group of cells.

Different concentrations of purified egg albumen NV sample are incubated with cells and their functional effects are evaluated by flow cytometry (Figure 4.12). NV samples are diluted with PBS and incubation duration was fixed as 1 hour. Untreated cells are negative controls and CI treated cells are positive control. 10 μ l of 1:100 diluted CD63 antibody was

used for staining for 30 minutes at room temperature in the dark, as previously determined. Interestingly, treatment with low concentration of albumen NV generated a mild activation in basophil cells, SI equals to 1.10, supporting our hypothesis. This activation is equivalent to 30% of 1 μ M CI activation. At higher concentration, albumen NV treatment produced advert effects, comparable to incubations with high CI content.

The efficiency of the CD203c staining was determined in a similar way with the CD63 antibody, using a dilution series of PE-conjugated CD203c antibody. Non-stained cells are compared with 5, 10 and 20 μ l 1:100 FACS buffer diluted CD203c antibody (Figure 4.13). Marked difference between unstained and stained samples implied the sensitivity and capability of the CD230c antibody.

The effects of albumen NV incubation on basophil cells were assessed using CD203c antibody, as in the CD63 antibody experiment (Figure 4.14). Albumen NV samples were further diluted to three different concentrations before incubation and staining processes were carried out as previously described. Our findings here confirmed our previous findings that the NV treatment induces basophil degranulation. Even more dramatically, the degranulation degree of the 1 μ l NV treated sample exceeded that of the positive control sample incubated with 1 μ M CI. While 0.5 μ l NV treatment exhibited minor activation, unfavorable effects similar to the previous experiment began to appear in the 1.5 μ l NV treated sample.

Interestingly, an increase in SSC-A is found in albumen NV treated samples, which corresponds to cell granularity. Differently, only the intensity of PE fluorescence increases in the CI-treated positive control (Figure 4.15). Similar to the phenomenon observed with the MFI, this effect has faded in larger concentrations of NV treatment sample. This could be due to physiological changes that occur in a brief period of time when a basophil cell is triggered. When the cells are stimulated by an allergen, they begin to degranulate and undergo particular cell state modifications. They eventually return to normal after stabilizing in their state. Significant granularity increase in NV treated samples could be attributed to increased granule synthesis in order to create a stronger immunological response.

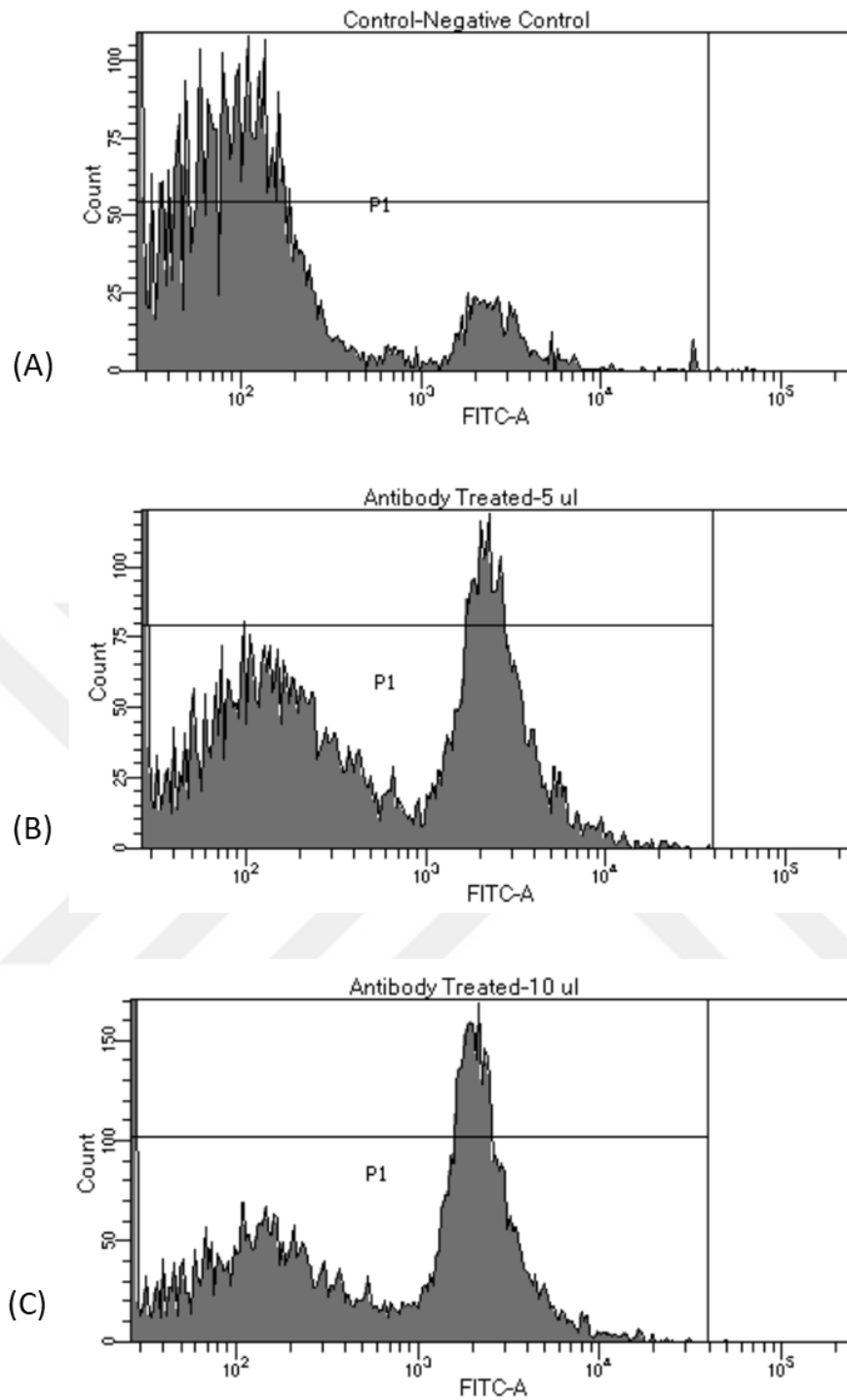


Figure 4.10: Flow cytometry CD63 antibody dilution series; (A) negative control without antibody treatment, MFI: 374, (B) 5 μ l antibody treatment, MFI: 959, (C) 10 μ l antibody treatment, MFI: 1170.

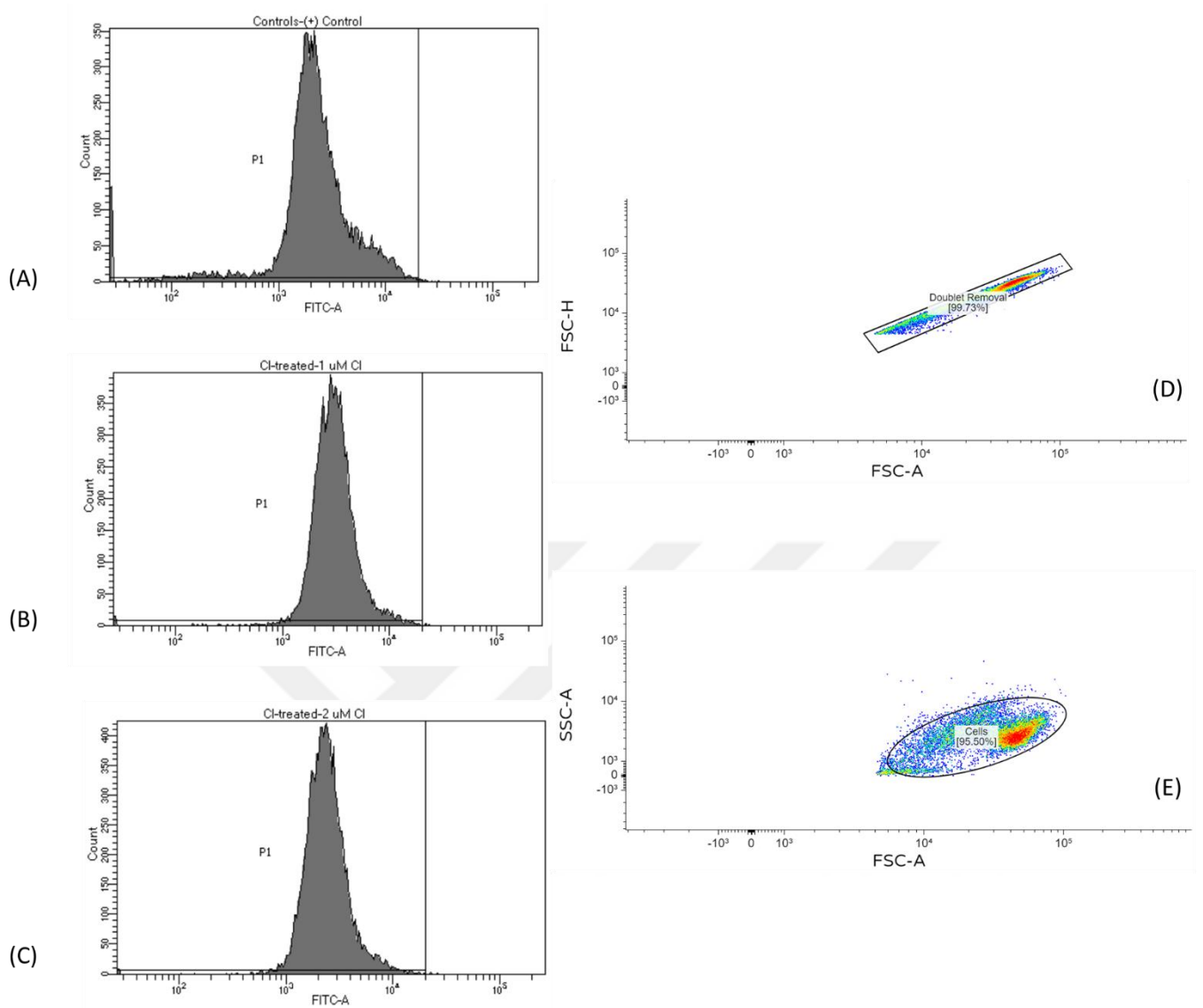


Figure 4.11: Flow cytometry CI activation with CD63 antibody; (A) untreated sample, MFI: 2922, (B) 1 μ M CI treatment, MFI: 3441, SI: 1.18, (C) 2 μ M CI treatment, MFI: 2773, (D) gating applied to the positive control sample to remove doublet cells, (E) gating applied to the positive control to filter out non-cell components such as debris.

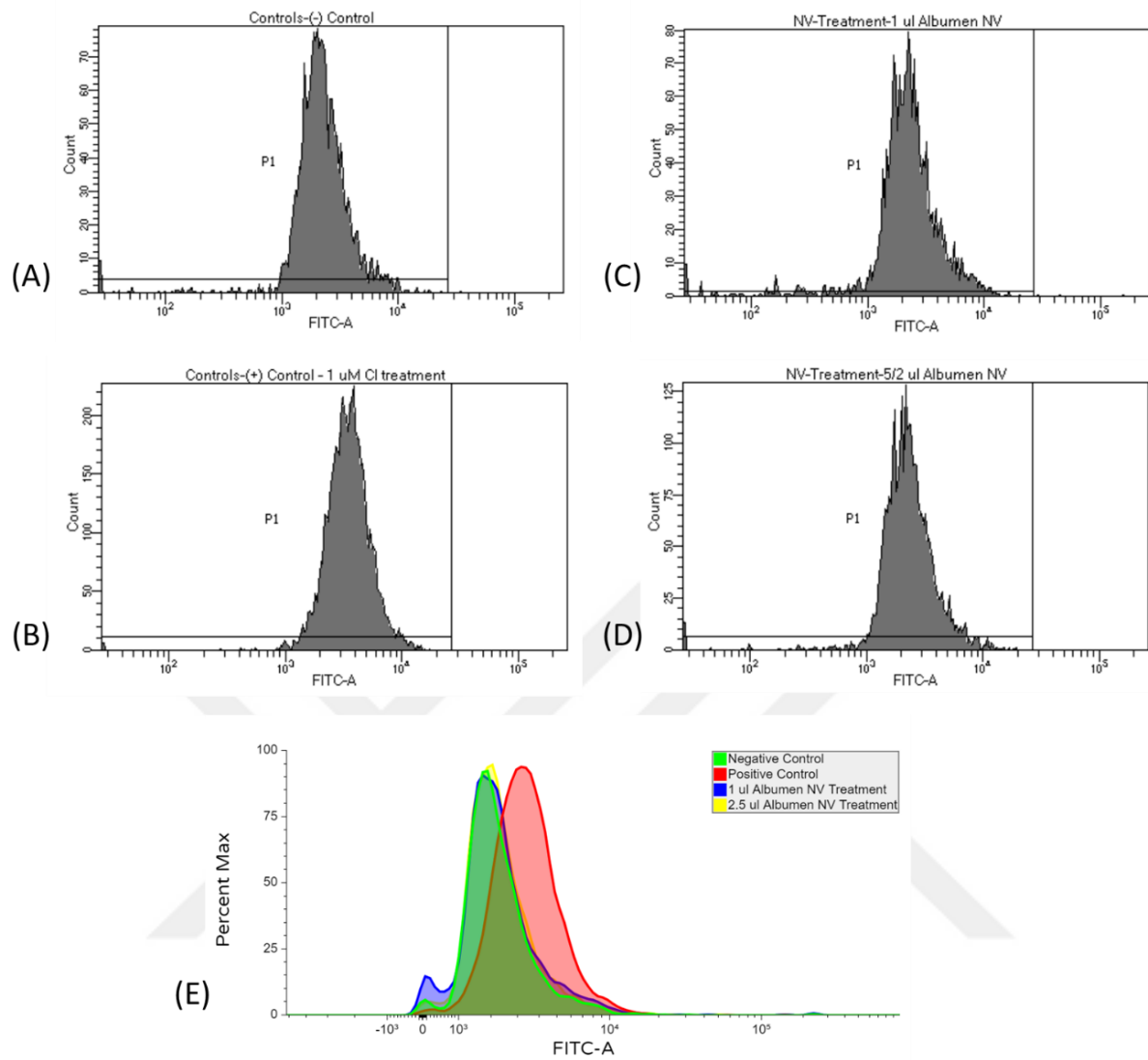


Figure 4.12: Flow cytometry with albumen nanovesicle transfection using CD63 antibody, (A) negative control - untreated sample, MFI: 2788, (B) positive control – 1 μ M CI treatment, MFI: 3756, SI: 1.35, (C) 1 μ L albumen NV treatment, MFI: 3069, SI: 1.10, (D) 2.5 μ l albumen NV treatment, MFI: 2626, (E) FITC-A count graph normalized according to the number of events.

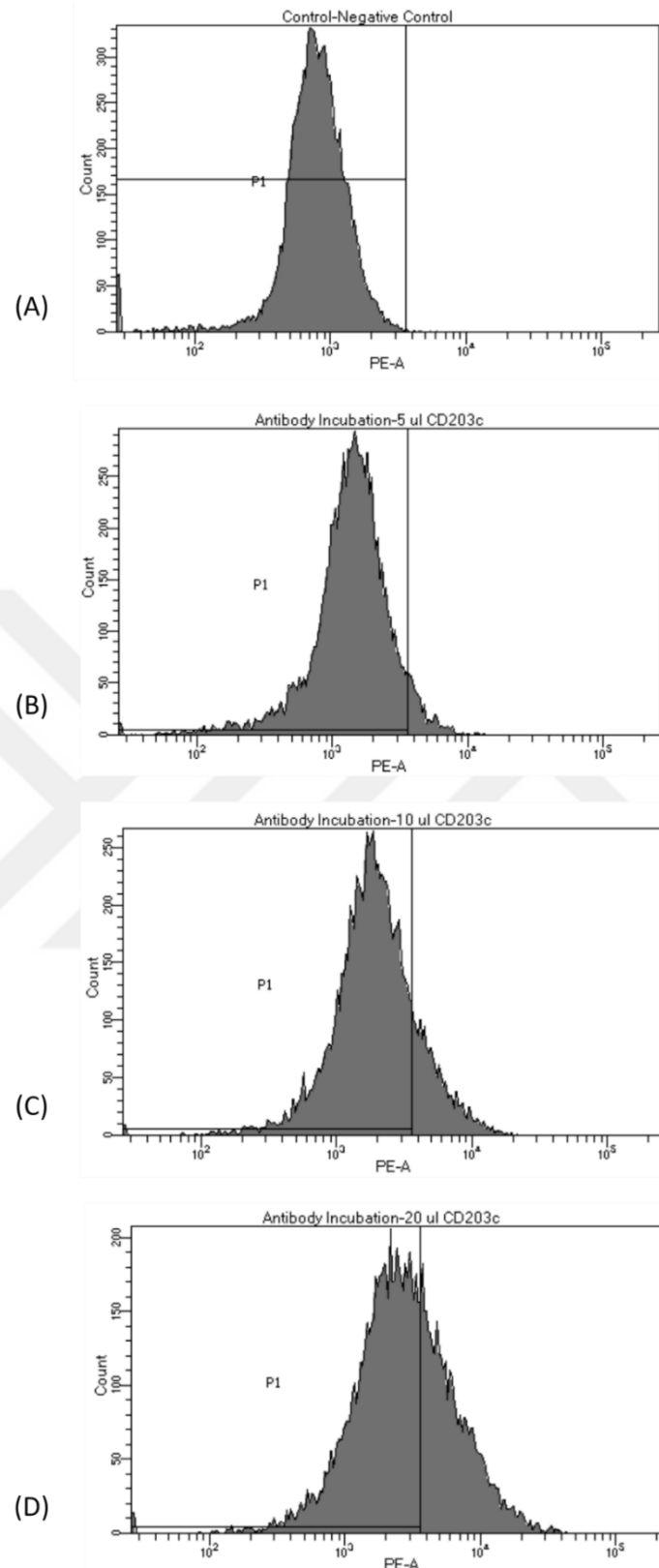


Figure 4.13: Flow cytometry CD203c antibody dilution series; (A) negative control without antibody treatment, MFI: 870, (B) 5 μ l antibody treatment, MFI: 1694, (C) 10 μ l antibody treatment, MFI: 2434, (D) 20 μ l antibody treatment, MFI: 3860.

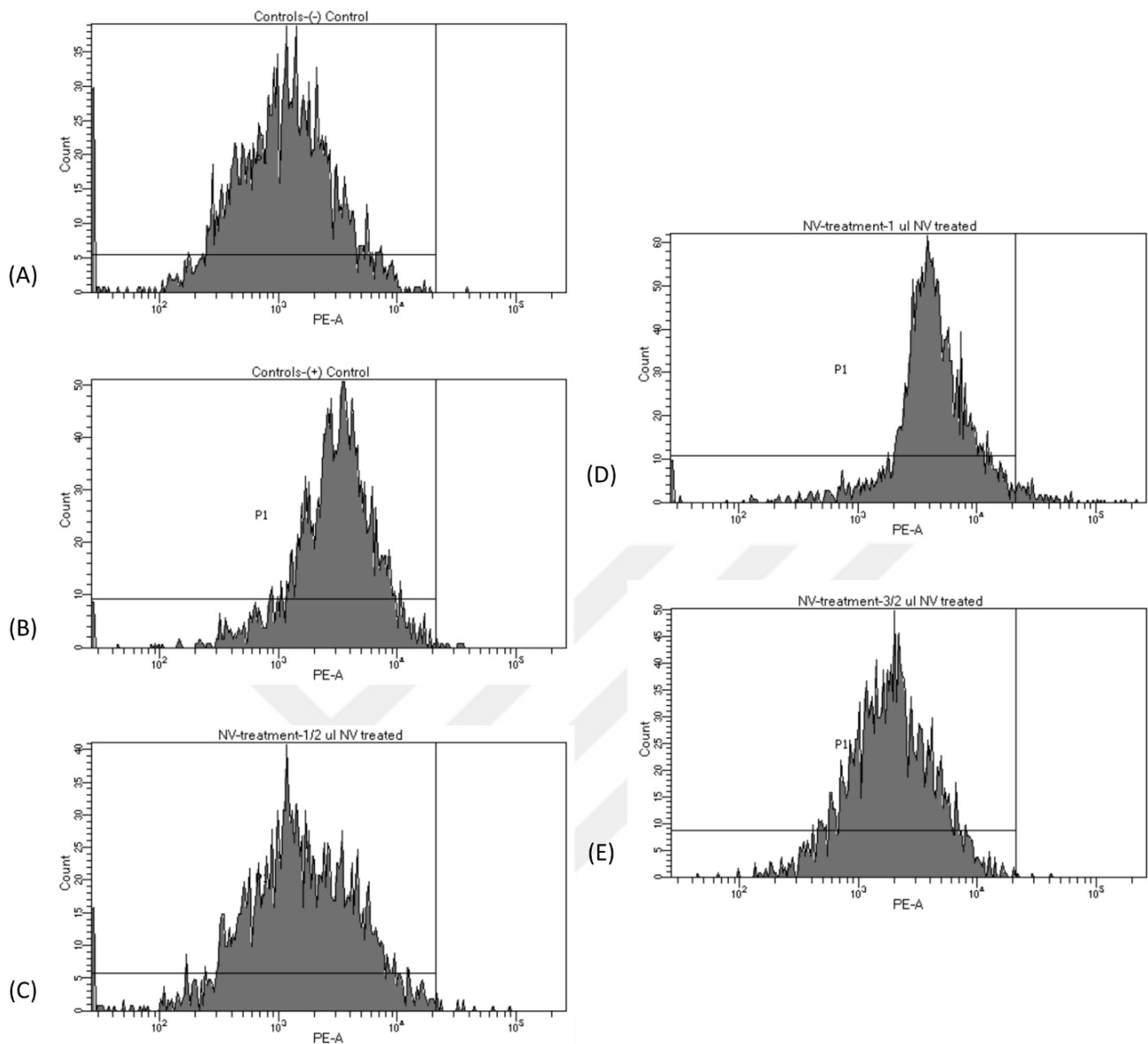


Figure 4.14: Flow cytometry with albumen nanovesicle transfection using CD203c antibody, (A) negative control - untreated sample, MFI: 1682, (B) positive control – 1 μ M CI treatment, MFI: 3284, SI: 1.95, (C) 0.5 μ L albumen NV treatment, MFI: 2717, SI: 1.61, (D) 1 μ L albumen NV treatment, MFI: 6337, SI: 3.77, (E) 1.5 μ L albumen NV treatment, MFI: 2535, SI: 1.51.

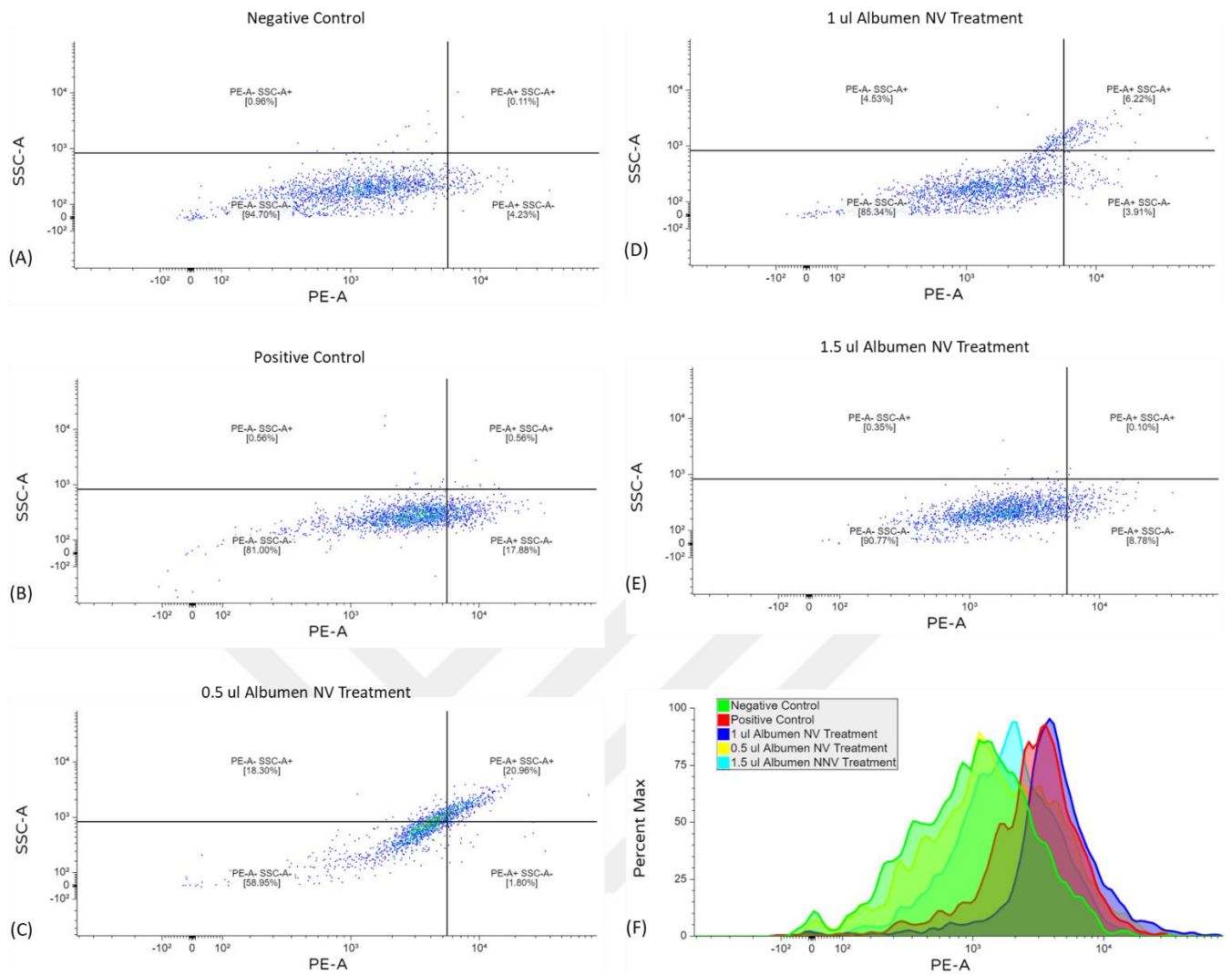


Figure 4.15: Flow cytometry with albumen nanovesicle transfection using CD203c antibody analysis, (A-E) PE-A vs SSC-A scatter plots of negative control, positive control, 0.5 ul albumen NV treatment, 1 ul albumen NV treatment, and 1.5 ul albumen NV treatment samples, (F) PE-A count graph normalized according to the number of events.

5 DISCUSSION

5.1 Prediction of Candidate Dietary Allergic miRNAs

MiRNA-target mRNA interactions are dynamic, complex, and difficult to predict because of several parameters influencing the outcome, including miRNA and target site abundance, subcellular localization, cell type/state, complementarity level, and miRISC stability (O'Brien et al, 2018). MiRNAs can target numerous genes at the same time, and it is thought that the majority of human coding genes are regulated by at least one miRNA. Most research relies on computational techniques to determine which genes might be targeted by a set of miRNAs; however, existing algorithms perform poorly in determining these interactions, resulting in numerous false positives (Liu & Wang, 2019). We used three miRNA-target algorithms with various operational mechanisms and combined their results to locate common miRNA-targets to reduce the number of false predictions and boost accuracy. As a result, we predicted that peanut miRNAs have the potential to influence 14 422 human genes, with 711 of these genes identified as DEGs in the T cells of food allergy patients (Figure 4.1). This figure is comparable to the 846 food allergy-related genes that might be affected by egg miRNAs (İnan, 2021). This demonstrates the potential of dietary miRNAs to influence allergy reactions in humans.

Bowtie alignment was used to determine the success of the small-RNA isolation and sequencing. The high proportion of clean readings and the high alignment rate show that the experimental techniques were successful (Table 4.1). Two techniques are used to determine which peanut miRNAs are most abundant in the samples. BLAST-plus performs a simple complementarity-based alignment. The results of two samples are comparable, and the top three most prevalent peanut miRNAs are identical but ranked differently (Appendix Table 7.4). To validate the BLAST-plus results, the miRDeep2 package is employed. It has been proposed as a highly powerful approach for both estimating known miRNAs and predicting novel miRNAs (Fridländer et al, 2012). Unlike BLAST-plus, the miRDeep2 algorithm indicated that only four of the peanut miRNAs were present in our sample, whereas the remaining thirteen peanut miRNAs could not be discovered. The miRDeep2 tool employs a highly stringent algorithm to determine the presence of miRNAs in reads, but it also performs an alignment based on basic sequence complementarity and provides it to the user alongside the algorithm results. Even though the miRDeep2 primary algorithm was unable to identify

the most common miRNAs identified by BLAST-plus, it admits that they are abundantly present in the data when assessed using the basic alignment technique. Because miRDeep2 algorithm requires the presence of both 5p and 3p mature miRNA products, it does not validate these miRNAs as existing in the sample and places them in a separate list. However, this could be due to the selection of one strand to be loaded onto a carrier protein or to be encapsulated into a vesicle. In that case, the other strand is rapidly degraded and could not be detected. Interestingly, miRDeep2 detected 215 novel peanut miRNAs, most of them having high total read counts (Appendix Table 7.2). According to its developers, miRDeep2 algorithm performs excellently in detecting novel miRNAs, with an accuracy higher than 99% (Friedländer et al., 2012). Considering this, our current knowledge on peanut miRNAs is severely poor and more research in this field is required.

It is critical to use computing tools to identify possibly allergenic dietary miRNAs before testing them in wet lab environments. In this regard, reducing the number of candidate miRNAs would benefit the implementer in the wet lab part. To accomplish this goal, the allergy hallmark genes targeted by the peanut and egg miRNAs were extracted from the data, and their influence quantities were calculated using the Reactome Pathway Browser (Table 4.4 & Table 4.5). Interestingly, some of the candidate miRNAs, ahy-miR-159a, ahy-miR482d-3p, gga-miR-1587, and gga-miR-2127, were detected as abundant in peanut according to our RNA-seq results, which may be correlated with their allergenic propensity.

5.2 Isolation Experiments

High-quality RNA extraction is usually problematic in plant molecular biology studies. Peanuts contain high levels of secondary metabolites such as tannins, lipids, polysaccharides, proteins, and several phenolic molecules (Huang et al., 2011). Conventional methods such as phenol-chloroform protocol were not suitable for the total RNA isolation from peanut; therefore, a procedure that uses CTAB as lysis buffer is employed with success. For the precipitation of the RNA from tissue lysate, different chemicals were tested to get the optimum results. To resuspend the precipitate, TE buffer effectively solubilizes the nucleic acids while formamide protects RNA molecules from degradation and enables long-term storage. Yet, the best results were obtained with overnight incubation of the sample with the

LiCl solution. By this method, even the smallest RNA molecules were collected in nut bud while maintaining their integrity (Figure 4.3).

Even though the high concentration of peanut total RNA at good purities were obtained using CTAB as lysis buffer and LiCl solution as precipitation agent, gel electrophoresis results showed that they were degraded. Performing experiments at strictly cold conditions, cleaning the bench area and equipment with ethanol, bleach and RNaseZAP solutions sequentially, addition of β -mercaptoethanol to the lysis buffer, collection of RNAs in formamide solution, did not improve the results. Nevertheless, total RNA isolation from freshly collected nut buds was performed successfully without degradation. Peanut samples used for the experiments were purchased from a local store, half roasted and packaged. Hence, it can be deduced that peanut RNAs might be inherently degraded even before the start of the experiments. To isolate high-quality RNA, peanuts could be collected directly from the live plants and transferred into liquid nitrogen to deactivate RNase enzymes. Even so, previous research from our group showed that miRNAs could be detected in degraded total RNA samples using stem-loop RT-PCR (Canik, 2022). This is consistent with the earlier studies indicating that the miRNAs are stable and resistant to degradative enzymes (Mitchell et al., 2008; O'Brien et al., 2018). In the light of these results, we hold out the hope to isolate miRNA from processed and packaged peanuts.

For nanovesicle isolation from peanut and egg, several protocols were employed to determine the best one (Table 3.3). According to the spectrophotometry results, the best method for the collection of nanovesicles is the protocol that combined ultracentrifugation with ultrafiltration. It is convenient for both peanut and egg samples working well with both. Nanovesicles at the final step are dissolved in PBS; however, at this step some difficulties arose with peanut nanovesicle. Some white particles remained in the solution suggesting that their physicochemical properties are not identical with egg nanovesicles. To overcome this, solubility of PBS can be enhanced by addition of small amounts of organic components such as HEPES or cholesterol.

If the presence of any impurity is determined in the nanovesicle samples, they can be purified using a sucrose cushion protocol with TCA precipitation successfully. The peak around 280 nm disappears with the aid of this procedure (Figure 4.4). This protocol's objective is to get rid of the contaminants while increasing the concentration of the nanovesicles. Accordingly, the nanovesicles are expected to be separated from other molecules based on their densities,

which is around 1.15 to 1.19 g/mL (Gupta, S., et al., 2018). However, the cushioning effect in our experiment was not that precise and nanovesicles were found to be spread into a few sucrose layers (Table 4.9 & Table 4.10). This could indicate the diversity of nanovesicles within a sample, related to density, size and content. It should also be noted that a fixed-angle ultracentrifuge rotor was used rather than a recommended swing-bucket rotor due to availability of instrument. This might have affected the segregation of the sucrose layers. Cooked egg nanovesicles accumulate in the upper fractions of the sucrose cushion, whereas albumen nanovesicles accumulate in the lower, denser portions. It is possible that the application of heat during the cooking process affects the structure and density of the nanovesicles. According to the literature, lipid nanovesicles are thermally stable, and even extreme temperatures cause only little structural deformation. However, they also point out that at specific temperatures organic molecules inside the nanovesicles might be combusted (Zacheo et al., 2020), which may explain changes in the structure and density in our cooked egg samples.

5.3 Characterization of Nanovesicle Samples

To analyze the purity, integrity, concentration and size distribution of the nanovesicle samples, various methods were utilized. Spectrophotometric analysis was performed primarily because of the ease of implementation and instant results with minimal wastage of sample. Additionally, presence of contaminants can be determined easily with the UV-Vis spectrum. Because of their phospholipid bilayer membrane, nanovesicles have an absorbance peak at 215 nm. At greater absorbances, such as 340 nm, the readout should decline dramatically, indicating that the sample is pure.

SEM imaging was performed to examine physical properties and appearance of nanovesicles. We used a simple overnight air-drying method on a glass slide, but some studies suggested usage of preservative agents such as glutaraldehyde and sodium cacodylate buffer to fix the nanovesicles and protect their structure and shape (De Robertis et al., 2020; Chen et al., 2019). Diversity in their size and shape is observed. In general, peanut nanovesicles are bigger in size, highly concentrated and have globular shapes systematically. On the other hand, egg yolk and albumen nanovesicles are smaller in size and highly disordered in their shape. Cooked egg nanovesicles were similar to raw egg samples with a dusty background.

The reason for that might be due to longer storage duration since this specific sample was acquired the previous year compared the other samples that are only one month old. Another reason also might be multiple freeze-thaw cycles that the samples undergone due to lack of consensus on at which temperature and how long nanovesicles could be stored. For long term storage -80 °C is suggested though there are studies proposing 4 and -20 °C also (Ly et al., 2023). Another factor affecting the quality of our nanovesicle samples could be multiple freeze-thaw cycles during the downstream applications. However, it is recommended to avoid freezing and thawing cycles since nanovesicles are structurally susceptible and it could harm their integrity (Yuan et al., 2021; Kim, K. et al., 2022).

NTA analysis was performed to measure the concentration of the nanovesicles as well as their size distribution. To be able to have accurate concentration measurement, samples were diluted. The high concentration of nanovesicles supports the findings from the spectrophotometric analysis. Size of nanovesicles changes mostly between 150-200 nm with bigger particles as 700 nm radius. This result is consistent with our SEM analysis, in which we determined an average radius of 136.5 nm for cooked egg nanovesicles. The size distribution of our egg nanovesicles is very similar to the ginseng-derived nanovesicles (Kim, J. et al., 2022), and to the *Endropanax morbifera* leaf-derived exosomes (Kim, K. et al., 2022). This size distribution, with a massive peak around 150-200 nm, could be characteristic of plant-derived nanovesicles.

5.4 Flow Cytometry for Functional Analysis

The human basophil cell line KU812 grows in RPMI-1640 cell media supplemented with FBS and Penicillin-Streptomycin. The removal of waste materials and residuals of dead cells by centrifugation, followed by the transfer of only healthy cells into fresh media, is crucial for high viability and the preservation of the culture's morphology. After thawing a vial of cells, it takes about a week for the cells to recover and resume normal reproduction. Based on these numerous parameters, the time required to perform functional analysis on cells should be carefully examined.

Flow cytometry is one of the most frequently used research techniques in immunological research. Basophil activation test (BAT) is a functional flow cytometry assay utilized in

clinical settings to detect basophil degranulation as a result of allergen contact in potentially allergic patients (Briceno Noriega et al., 2021). For our study, the two most popular activation markers, CD63 and CD203c, are selected. CD63 antibody was conjugated with fluorescein isothiocyanate (FITC) while CD203c was attached to a phycoerythrin (PE) fluorophore.

A dilution series with antibodies was performed to determine the most suitable antibody concentration (Figure 4.10). Binding of the FITC-conjugated antibody is obvious even in the lowest concentration, 5 μ l, compared to the negative control that is not stained. In the 20 μ l antibody incubation, a cell pellet was lost during the washing process which could explain the disruption of consistency within the samples. 10 μ l antibody for 1×10^5 cells/100 μ l has been decided to be used for further experiments.

CI is a chemical that has long been recognized for inducing granulocyte degranulation and is commonly employed as a control in basophil and mast cell activation assays (Millipore, 2008). On KU812 cells, the effects of various CI concentrations and incubation times are investigated. The 1-hour incubation with a final CI concentration of 100 nanomolar is determined to be the most substantial activation (Figure 4.11). Higher CI concentrations may cause undesirable consequences such as rapid degranulation and return to normal cell state in a shorter amount of time.

The nanovesicle samples were diluted before transfection with the cells because they were too concentrated. Albumen NV treatment for 1-hour at 37 °C induces a minor activation in the basophil cells as compared to the negative control sample (Figure 4.12). Although not as strong as 1 μ M CI activation, NV treatment achieves around 30% of CI activation level when mean fluorescence intensities are compared. Samples transfected with higher concentrations of albumen NV showed similar adverse effects as the high concentration of CI treatment. These findings suggest that food derived nanovesicles may increase allergic reactions in human basophil cells by inducing degranulation.

PE-conjugated CD203c antibody was used at different concentrations to stain the basophil cells. The staining was effective even with the lowest antibody concentration (Figure 4.13). Same concentration as CD63 antibody, 10 μ l antibody solution per 200 μ l of sample volume, was set for further experiments.

We diluted albumen NV samples further and used a more limited concentration range with a larger number of samples based on the NV treatment findings with CD63 antibody. Our results here validated our prior findings that NV treatment causes basophil degranulation. Even more impressively, the degree of degranulation in the 1 μ l NV-treatment group was greater compared to the 1 μ M CI treated sample. We also observed a larger degranulation level as the concentration of albumen NV incubation increased; nevertheless, the sample with the highest concentration of NV treatment began to exhibit counter effects. This was also observed with the higher concentrations of CI treatment (Figure 4.14). The basophil activation test is commonly utilized for diagnostic purposes in clinical settings as well as for immunological disease research, including peanut and egg allergies. The basophil activation test targeting CD63 and CD203c revealed an excellent performance for the diagnosis of peanut and egg allergies with accuracy higher than 90% and sensitivity exceeding than 95% (Santos et al., 2014; Ocmant et al., 2009), verifying the reliability of our flow cytometry findings.

Another interesting finding was that the increase in SSC-A cell granularity component in the albumen NV treated samples (Figure 4.15). This effect was specific to NV treated samples and was not observed in the CI treated positive control. This could be related to the activation process, which induces physiological changes in the cell state; however, more research is needed to understand how this occurs.

When FITC-conjugated CD63 antibodies and PE-conjugated CD203c antibodies are compared, PE is found to be more sensitive. Basophil cells treated with equivalent amounts of albumen nanovesicles, 1 μ l, had a SI of 3.77 when stained with CD230c antibody, but only 1.10 when stained with CD63 antibody. Similarly, Zhang *et al.* demonstrated that PE surpassed FITC in the detection of a surface protein in microglia (2022). Furthermore, FITC is very sensitive to pH alterations that may occur as a result of the degranulation of basophilic granules (Bangs Laboratories, Inc., 2017). To understand the underlying cause of antibody differences, the behaviors of each fluorophore and marker in these conditions should be evaluated separately. To accomplish this goal, we could use PE-conjugated CD63 and FITC-conjugated CD203c antibodies; this would allow us to determine if the variance among samples was attributable to the fluorophore used or something inherent to the targeted marker.

5.5 Future Prospects

In this study, we hypothesize that dietary miRNAs encapsulated in lipid nanovesicles may trigger allergic reactions due to their ability to penetrate deeper in the human gastrointestinal tract and their immunomodulatory potential. We presented preliminary findings that supported our theory; nevertheless, more research must be conducted to confirm the role of miRNAs in food allergy, recommended as follows:

- FITC-conjugated CD63 and PE-conjugated CD203c flow cytometry experiments were performed. To investigate the underlying cause of antibodies' varied responses, a co-staining experiment using both antibodies could be conducted. To validate our results, further flow cytometry settings utilizing IgE antibodies could be performed.
- Only albumen nanovesicles were used in functional assays on basophil cell lines. Using high-quality peanut nanovesicle samples in hand, identical flow cytometry experiments could be replicated to assess if our basophil cells react in a similar manner to nanovesicles from different food sources.
- There are commercial kits created specifically to determine granulocyte degranulation. The mast cell degranulation assay and histamine assay kits were purchased to validate the flow cytometry results by assessing different parameters. Mast cell degranulation assay determines the quantity of tryptase enzyme released from basophil granules, whereas histamine assay determines the amount of histamine. These assays are being optimized, and they can be further expanded to study the impact of nanovesicles and miRNAs on food allergies.
- To gain a deeper understanding of the miRNAs inside the nanovesicles, small RNA isolation from the nanovesicle samples could be performed, followed by a sequencing and RNA-seq analysis. Thus, miRNAs localized in the nanovesicles and in the cytoplasm can be compared.
- Candidate allergenic miRNAs were determined based on their target genes using computational techniques. Here, I narrow down the list of candidate miRNAs to two for each food source (Table 5.1). These miRNAs could be manufactured synthetically, encapsulated inside lipid nanovesicles with Lipofectamine, and transfected into the KU812 basophil cell line. As a result, the impact of specific miRNAs on allergies can be assessed.

- In a continuous cell culture, toluidine blue solutions are utilized to detect basophil granules under the microscope. In this way, microscopic investigation can reveal basophil activation and degranulation.

Table 5.1: Candidate peanut and egg miRNAs for future prospects.

	miRNA Name	Sequence
Peanut	ahy-miR-159a	UUGGAUUGAAGGGAGCUCUA
	ahy-miR482d-3p	UCUUCCCUACACCUCCCAUACC
Egg	gga-miR-1587	GGGCUGGGCUGGGCUGGGCA
	gga-miR-2127	CGGGAGGGGAGGGAGGGCGGG

6 BIBLIOGRAPHY

- Anvari, S., Miller, J., Yeh, C., & Davis, C. (2018). IgE-Mediated Food Allergy. *Clinical Reviews In Allergy & Immunology*, 57(2), 244-260. doi: 10.1007/s12016-018-8710-3
- Aquilano, K., Ceci, V., Gismondi, A., De Stefano, S., Iacovelli, F., & Faraonio, R. et al. (2019). Adipocyte metabolism is improved by TNF receptor-targeting small RNAs identified from dried nuts. *Communications Biology*, 2(1). doi: 10.1038/s42003-019-0563-7
- ATCC CRL-2099. (2023). *KU812 | ATCC Product Sheet*.
- Bangs Laboratories, Inc. (2017). FITC / PE Compensation Standard | *Product Data Sheet* 820.
- Bergmann, R.L., Diepgen, T.L., Kuss, O., Bergmann, K.E., Kujat, J., Dudenhausen, J.W., & Wahn, U. (2002). Breastfeeding duration is a risk factor for atopic eczema. *Clinical & Experimental Allergy*, 32: 205-209. doi: 10.1046/j.1365-2222.2002.01274.x
- Briceno Noriega, D., Teodorowicz, M., Savelkoul, H., & Ruinemans-Koerts, J. (2021). The Basophil Activation Test for Clinical Management of Food Allergies: Recent Advances and Future Directions. *Journal Of Asthma And Allergy*, Volume 14, 1335-1348. doi: 10.2147/jaa.s237759
- Briceno Noriega, D., Teodorowicz, M., Savelkoul, H., & Ruinemans-Koerts, J. (2021). The Basophil Activation Test for Clinical Management of Food Allergies: Recent Advances and Future Directions. *Journal Of Asthma And Allergy*, Volume 14, 1335-1348. doi: 10.2147/jaa.s237759
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. (2009). BLAST+: architecture and applications. *BMC Bioinformatics*, 10(1). doi: 10.1186/1471-2105-10-421
- Canik, O.I.. (2022). Identification of risk factors and biomarkers for food allergy from ingested miRNAs [Master's thesis, Sabanci University]
- Chen, X., Zhou, Y., & Yu, J. (2019). Exosome-like Nanoparticles from Ginger Rhizomes Inhibited NLRP3 Inflammasome Activation. : *Mol. Pharmaceutics*, 16, 2690–2699. doi: 10.1021/acs.molpharmaceut.9b00246
- Choudhary, O. P., & Priyanka. (2017). Scanning Electron Microscope: Advantages and Disadvantages in Imaging Components. *International Journal Of Current*

- Microbiology And Applied Sciences, 6(5), 1877-1882. doi: 10.20546/ijcmas.2017.605.207
- Comfort, N., Cai, K., Bloomquist, T., Strait, M., Ferrante Jr., A., & Baccarelli, A. (2021). Nanoparticle Tracking Analysis for the Quantification and Size Determination of Extracellular Vesicles. *Journal Of Visualized Experiments*, (169). doi: 10.3791/62447
- Cui, M., Wang, H., Yao, X., Zhang, D., Xie, Y., Cui, R., & Zhang, X. (2019). Circulating MicroRNAs in Cancer: Potential and Challenge. *Frontiers In Genetics*, 10. doi: 10.3389/fgene.2019.00626
- Dantas, M., Tenório, H., Lopes, T., Pereira, H., Marsaioli, A., Figueiredo, I., & Santos, J. (2017). Interactions of tetracyclines with ovalbumin, the main allergen protein from egg white: Spectroscopic and electrophoretic studies. *International Journal Of Biological Macromolecules*, 102, 505-514. doi: 10.1016/j.ijbiomac.2017.04.052
- De Robertis, M., Sarra, A., D'Oria, V., Mura, F., Bordi, F., Postorino, P., & Fratantonio, D. (2020). Blueberry-Derived Exosome-Like Nanoparticles Counter the Response to TNF- α -Induced Change on Gene Expression in EA.hy926 Cells. *Biomolecules*, 10(5), 742. doi: 10.3390/biom10050742
- Docena, G.H., Fernandez, R., Chirido ,F.G., & Fossati, C.A. (1996). Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy*, 51: 412-416. doi: 10.1111/j.1398-9995.1996.tb04639.x
- Doyle, J. J. and Doyle J. L. A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue *Phytochemical Bulletin*, Vol. 19, 1987, pp. 11-15.
- Dragovic, R., Gardiner, C., Brooks, A., Tannetta, D., Ferguson, D., & Hole, P. et al. (2011). Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis. *Nanomedicine: Nanotechnology, Biology And Medicine*, 7(6), 780-788. doi: 10.1016/j.nano.2011.04.003
- Ebeling, W., Hennrich, N., Klockow, M., Metz, H., Orth, H. D., & Lang, H. (1974). Proteinase K from *Tritirachium album* Limber. *European Journal of Biochemistry*, 47, 91-97. doi: 10.1111/j.1432-1033.1974.tb03671.x
- Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., & Marks, D. S. (2003). MicroRNA targets in *Drosophila*. *Genome Biology*, 5(1), 1. doi: 10.1186/gb-2003-5-1-r1
- Fernandez-Rivas, M., Vereda, A., Vickery, B., Sharma, V., Nilsson, C., & Muraro, A. et al. (2021). Open-label follow-on study evaluating the efficacy, safety, and quality of life with extended daily oral immunotherapy in children with peanut allergy. *Allergy*, 77(3), 991-1003. doi: 10.1111/all.15027

- Ferraz, T., Fiúza, M., dos Santos, M., Pontes de Carvalho, L., & Soares, N. (2004). Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. *Journal Of Biochemical And Biophysical Methods*, 58(3), 187-193. doi: 10.1016/j.jbbm.2003.10.008
- Friedländer, M., Mackowiak, S., Li, N., Chen, W., & Rajewsky, N. (2012). miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Research*, 40(1), 37-52. doi: 10.1093/nar/gkr688
- Gerrard, J.W., Vickers, P., & Gerrard, C.D. (1976). The familial incidence of allergic disease. *Annals of Allergy*. 36(1):10-15. PMID: 1247187.
- Gillespie, M., Jassal, B., Stephan, R., Milacic, M., Rothfels, K., & Senff-Ribeiro, A. et al. (2021). The reactome pathway knowledgebase 2022. *Nucleic Acids Research*, 50(D1), D687-D692. doi: 10.1093/nar/gkab1028
- Gupta, R., Warren, C., Smith, B., Blumenstock, J., Jiang, J., Davis, M., & Nadeau, K. (2018). The Public Health Impact of Parent-Reported Childhood Food Allergies in the United States. *Pediatrics*, 142(6). doi: 10.1542/peds.2018-1235
- Gupta, S., Rawat, S., Arora, V., Kottarath, S., Dinda, A., & Vaishnav, P. et al. (2018). An improvised one-step sucrose cushion ultracentrifugation method for exosome isolation from culture supernatants of mesenchymal stem cells. *Stem Cell Research & Therapy*, 9(1). doi: 10.1186/s13287-018-0923-0
- Hamam, R., Hamam, D., Alsaleh, K., Kassem, M., Zaher, W., & Alfayez, M. et al. (2017). Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers. *Cell Death & Disease*, 8(9), e3045-e3045. doi: 10.1038/cddis.2017.440
- He, J., Zhao, J., Zhu, W., Qi, D., Wang, L., & Sun, J. et al. (2016). MicroRNA biogenesis pathway genes polymorphisms and cancer risk: a systematic review and meta-analysis. *Peerj*, 4, e2706. doi: 10.7717/peerj.2706
- Hourihane, J., Dean, T., & Warner, J. (1996). Peanut allergy in relation to heredity, maternal diet, and other atopic diseases: results of a questionnaire survey, skin prick testing, and food challenges. *BMJ*, 313(7056), 518-521. doi: 10.1136/bmj.313.7056.518
- Huang, C., Picimbon, J., Li, H., Li, Z., Liu, Q., & Liu, W. (2011). An efficient method for total RNA extraction from peanut seeds. *Russian Journal Of Plant Physiology*, 59(1), 129-133. doi: 10.1134/s1021443712010074
- Hwang, D., Nagler, C., & Ciaccio, C. (2022). New and emerging concepts and therapies for the treatment of food allergy. *Immunotherapy Advances*, 2(1). doi: 10.1093/immadv/ltac006

- İnan, A. (2021). In silico analysis of the food allergy related pathways and the potential role of dietary miRNAs [Master's thesis, Sabanci University].
- Kabashima, K, Nakashima, C, Nonomura, Y, Otsuka, A., Cardamone, C., Parente, R., De Feo, G., & Triggiani, M. (2018). Biomarkers for evaluation of mast cell and basophil activation. *Immunol Rev.* 282: 114– 120. doi: 10.1111/imr.12639
- Kim, J., Lee, Y., Wang, J., Kim, Y., & Kwon, I. (2022). Isolation and characterization of ginseng-derived exosome-like nanoparticles with sucrose cushioning followed by ultracentrifugation. *SN Applied Sciences*, 4(2). doi: 10.1007/s42452-022-04943-y
- Kim, K., Park, J., Sohn, Y., Oh, C., Park, J., Yuk, J., & Yeon, J. (2022). Stability of Plant Leaf-Derived Extracellular Vesicles According to Preservative and Storage Temperature. *Pharmaceutics*, 14(2), 457. doi: 10.3390/pharmaceutics14020457
- Kozomara, A., Birgaoanu, M., & Griffiths-Jones, S. (2018). miRBase: from microRNA sequences to function. *Nucleic Acids Research*, 47(D1), D155-D162. doi: 10.1093/nar/gky1141
- Lack, G. (2008). Epidemiologic risks for food allergy. *Journal Of Allergy And Clinical Immunology*, 121(6), 1331-1336. doi: 10.1016/j.jaci.2008.04.032
- Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*, 10(3), R25. doi: 10.1186/gb-2009-10-3-r25
- Leinonen, R., Sugawara, H., & Shumway, M. (2011). The Sequence Read Archive. *Nucleic Acids Research*, 39(Database issue), D19. doi: 10.1093/NAR/GKQ1019
- Liu, W., & Wang, X. (2019). Prediction of functional microRNA targets by integrative modeling of microRNA binding and target expression data. *Genome Biology*, 20(1). doi: 10.1186/s13059-019-1629-z
- Loh, W., & Tang, M. (2018). The Epidemiology of Food Allergy in the Global Context. *International Journal Of Environmental Research And Public Health*, 15(9), 2043. doi: 10.3390/ijerph15092043
- Ly, N., Han, H., Kim, M., Park, J., & Choi, K. (2023). Plant-derived nanovesicles: Current understanding and applications for cancer therapy. *Bioactive Materials*, 22, 365-383. doi: 10.1016/j.bioactmat.2022.10.005
- MacFarlane, L., & R. Murphy, P. (2010). MicroRNA: Biogenesis, Function and Role in Cancer. *Current Genomics*, 11(7), 537-561. doi: 10.2174/138920210793175895

- Martino, D., Neeland, M., Dang, T., Cobb, J., Ellis, J., & Barnett, A. et al. (2018). Epigenetic dysregulation of naive CD4⁺ T-cell activation genes in childhood food allergy. *Nature Communications*, 9(1). doi: 10.1038/s41467-018-05608-4
- Matsui, T., Tanaka, K., Yamashita, H., Saneyasu, K., Tanaka, H., & Takasato, Y. et al. (2019). Food allergy is linked to season of birth, sun exposure, and vitamin D deficiency. *Allergology International*, 68(2), 172-177. doi: 10.1016/j.alit.2018.12.003
- McGeary, S., Lin, K., Shi, C., Pham, T., Bisaria, N., Kelley, G., & Bartel, D. (2019). The biochemical basis of microRNA targeting efficacy. *Science*, 366(6472). doi: 10.1126/science.aav1741
- Millipore. (2008). Mast Cell Degranulation Assay Kit - IMM001 User Guide.
- Misiak, R.T., Wegienka, G., Havstad, S., et al. (2009). Specific allergic sensitization in parents and their 18-year-old offspring in the Suburban Detroit Childhood Allergy Study. *The Journal of Allergy and Clinical Immunology*. 123(6):1401-6.e2. doi: 10.1016/j.jaci.2009.03.006.
- Mitchell, P., Parkin, R., Kroh, E., Fritz, B., Wyman, S., & Pogosova-Agadjanyan, E. et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings Of The National Academy Of Sciences*, 105(30), 10513-10518. doi: 10.1073/pnas.0804549105
- Moriyama, T. (2015). Diversity of Food Allergy. *Journal Of Nutritional Science And Vitaminology*, 61(Supplement), S106-S108. doi: 10.3177/jnsv.61.s106
- Mueller, G., Maleki, S., & Pedersen, L. (2014). The Molecular Basis of Peanut Allergy. *Current Allergy And Asthma Reports*, 14(5). doi: 10.1007/s11882-014-0429-5
- O'Brien, J., Hayder, H., Zayed, Y., & Peng, C. (2018). Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Frontiers In Endocrinology*, 9. doi: 10.3389/fendo.2018.00402
- Ocmant, A., Mulier, S., Hanssens, L., Goldman, M., Casimir, G., Mascart, F., & Schandené, L. (2009). Basophil activation tests for the diagnosis of food allergy in children. *Clinical & Experimental Allergy*, 39, 1234–1245. doi: 10.1111/j.1365-2222.2009.03292.x
- Prescott, S., & Allen, K.J. (2011) Food allergy: Riding the second wave of the allergy epidemic. *Pediatric Allergy and Immunology*; 22: 155–16. doi: 10.1111/j.1399-3038.2011.01145.x
- R Core Team. (2023). R: A Language and Environment for Statistical Computing. Foundation for Statistical Computing, Vienna, Austria.

- Redrup, A.C., Howard, B.P., MacGlashan, D.W., Jr Kagey-Sobotka, A., Lichtenstein, L.M., & Schroeder, J.T. (1998). Differential regulation of IL-4 and IL-13 secretion by human basophils: their relationship to histamine release in mixed leukocyte cultures. *J Immunol.* 15;160(4): 1957-64. doi: 10.4049/jimmunol.160.4.1957
- Rehmsmeier, M., Steffen, P., Höschmann, M., & Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. *RNA*, 10(10), 1507-1517. doi: 10.1261/rna.5248604
- Rider, M., Hurwitz, S., & Meckes, D. (2016). ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles. *Scientific Reports*, 6(1). doi: 10.1038/srep23978
- Salloum-Asfar, S., Satheesh, N., & Abdulla, S. (2019). Circulating miRNAs, Small but Promising Biomarkers for Autism Spectrum Disorder. *Frontiers In Molecular Neuroscience*, 12. doi: 10.3389/fnmol.2019.00253
- Sampson, H. (1999). Food allergy. Part 2: Diagnosis and management. *Journal Of Allergy And Clinical Immunology*, 103(6), 981-989. doi: 10.1016/s0091-6749(99)70167-3
- Santos, A., Douiri, A., Bécares, N., Wu, S., Stephens, A., & Radulovic, S. et al. (2014). Basophil activation test discriminates between allergy and tolerance in peanut-sensitized children. *Journal Of Allergy And Clinical Immunology*, 134(3), 645-652. doi: 10.1016/j.jaci.2014.04.039
- Schmutz, J., Jenkins, J., Grimwood, J., Bertoli, D.J., Leal-Bertoli, S., Clevenger, J., Micheltore, R., Froenke, L., Cannon, S.B., Varshney, R., Schleffler, B., Jackson, S.A. & Ozias-Akins, P. (2018). The *Arachis hypogaea* whole genome shotgun (WGS) project. *Agronomy*. Accession number: PIVG00000000
- Schneider, C., Rasband, W., & Eliceiri, K. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671-675. doi: 10.1038/nmeth.2089
- Schneider, V., Graves-Lindsay, T., Howe, K., Bouk, N., Chen, H., & Kitts, P. et al. (2016). Evaluation of GRCh38 and de novo haploid genome assemblies demonstrates the enduring quality of the reference assembly. doi: 10.1101/072116
- Schoos, A., Bullens, D., Chawes, B., Costa, J., De Vlieger, L., & DunnGalvin, A. et al. (2020). Immunological Outcomes of Allergen-Specific Immunotherapy in Food Allergy. *Frontiers In Immunology*, 11. doi: 10.3389/fimmu.2020.568598
- Sicherer, S., & Sampson, H. (2014). Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *Journal Of Allergy And Clinical Immunology*, 133(2), 291-307.e5. doi: 10.1016/j.jaci.2013.11.020

- Sicherer, S., Warren, C., Dant, C., Gupta, R., & Nadeau, K. (2020). Food Allergy from Infancy Through Adulthood. *The Journal Of Allergy And Clinical Immunology: In Practice*, 8(6), 1854-1864. doi: 10.1016/j.jaip.2020.02.010
- Siracusa, M., Kim, B., Spergel, J., & Artis, D. (2013). Basophils and allergic inflammation. *Journal Of Allergy And Clinical Immunology*, 132(4), 789-801. doi: 10.1016/j.jaci.2013.07.046
- Thermo Scientific. (2010). Nalgene® Mr. Frosty® Cryo 1°C Freezing Containers Manual.
- Toni, L., Garcia, A., Jeffrey, D., Jiang, X., Stauffer, B., Miyamoto, S., & Sucharov, C. (2018). Optimization of phenol-chloroform RNA extraction. *Methodsx*, 5, 599-608. doi: 10.1016/j.mex.2018.05.011
- Turnbull, J.L., Adams, H.N. and Gorard, D.A. (2014) Review article: The diagnosis and management of of food allergy and food intolerances. *Alimentary Pharmacology & Therapeutics*, 41: 3-25. doi:10.1111/apt.12984
- Van de Vlekkert, D., Qiu, X., Annunziata, I., & d'Azzo, A. (2020). Isolation, Purification and Characterization of Exosomes from Fibroblast Cultures of Skeletal Muscle. *BIO-PROTOCOL*, 10(7). doi: 10.21769/bioprotoc.3576
- Vickery, B.P., Vereda, A., Casale, T.B., Beyer, K., Du Toit, C., Hourihane, J.O., Jones, S.M., Shreffler, W.G. et al. (2018). AR101 Oral Immunotherapy for Peanut Allergy. *The New England Journal of Medicine*, 379:1991-2001. doi: 10.1056/NEJMoa1812856
- Waserman, S., Bégin, P., & Watson, W. (2018). IgE-mediated food allergy. *Allergy, Asthma & Clinical Immunology*, 14(S2). doi: 10.1186/s13223-018-0284-3
- Weidner, J., Bartel, S., Kılıç, A., Zissler, U., Renz, H., & Schwarze, J. et al. (2020). Spotlight on microRNAs in allergy and asthma. *Allergy*, 76(6), 1661-1678. doi: 10.1111/all.14646
- Xu, W., Jiang, X., & Huang, L. (2019). RNA Interference Technology. *Comprehensive Biotechnology*, 560-575. doi: 10.1016/b978-0-444-64046-8.00282-2
- Yuan, F., Li, Y., & Wang, Z. (2021). Preserving extracellular vesicles for biomedical applications: consideration of storage stability before and after isolation. *Drug Delivery*, 28(1), 1501-1509. doi: 10.1080/10717544.2021.1951896
- Zacheo, A., Bizzarro, L., Blasi, L., Piccirillo, C., Cardone, A., & Gigli, G. et al. (2020). Lipid-Based Nanovesicles for Simultaneous Intracellular Delivery of Hydrophobic, Hydrophilic, and Amphiphilic Species. *Frontiers In Bioengineering And Biotechnology*, 8. doi: 10.3389/fbioe.2020.00690

- Zhang, H., Tan, C., Shi, X., & Xu, J. (2022). Impacts of autofluorescence on fluorescence based techniques to study microglia. *BMC Neuroscience*, 23(1). doi: 10.1186/s12868-022-00703-1
- Zhang, Y., & Verbeek, F.J. (2010). Comparison and integration of target prediction algorithms for microRNA studies. *Journal of Integrative Bioinformatics*, 7(3):127. doi: 10.2390/biecoll-jib-2010-127
- Żukiewicz-Sobczak, W., Wróblewska, P., Adamczuk, P., & Kopczyński, P. (2013). Causes, symptoms and prevention of food allergy. *Advances In Dermatology And Allergology*, 2, 113-116. doi: 10.5114/pdia.2013.34162



7 APPENDIX

Mature Peanut miRNA Sequences from miRBase

>ahy-miR156a MIMAT0016317 Arachis hypogaea miR156a
UGACAGAAGAGAGAGAGCAC

>ahy-miR156b-5p MIMAT0016318 Arachis hypogaea miR156b-5p
UUGACAGAAGAUAGAGAGCAC

>ahy-miR156b-3p MIMAT0016319 Arachis hypogaea miR156b-3p
GCUCUCUAAGCUUCUGUCAUC

>ahy-miR156c MIMAT0016320 Arachis hypogaea miR156c
UUGACAGAAGAGAGAGAGCAC

>ahy-miR159 MIMAT0016321 Arachis hypogaea miR159
UUUGGAUUGAAGGGAGCUCUA

>ahy-miR160-5p MIMAT0016322 Arachis hypogaea miR160-5p
UGCCUGGCUCCCUGAAUGCCA

>ahy-miR160-3p MIMAT0016323 Arachis hypogaea miR160-3p
GCAUGAAGGGAGUCACGCAGG

>ahy-miR167-5p MIMAT0016324 Arachis hypogaea miR167-5p
UGAAGCUGCCAGCAUGAUCUU

>ahy-miR167-3p MIMAT0016325 Arachis hypogaea miR167-3p
AGAUCAUGUGGCAGUUUCACC

>ahy-miR394 MIMAT0016326 Arachis hypogaea miR394
UUGGCAUUCUGUCCACCUC

>ahy-miR398 MIMAT0016327 Arachis hypogaea miR398
UGUGUUCUCAGGUCACCCCU

>ahy-miR408-5p MIMAT0016328 Arachis hypogaea miR408-5p
CUGGGAACAGGCAGAGCAUGA

>ahy-miR408-3p MIMAT0016329 Arachis hypogaea miR408-3p
AUGCACUGCCUCUUCCCUGGC

>ahy-miR3508 MIMAT0016330 Arachis hypogaea miR3508
UAGAGGGUCCCCAUGUUCUCA

>ahy-miR3509-5p MIMAT0016331 Arachis hypogaea miR3509-5p
AUACUUGAGAGCCGUAGAUGA

>ahy-miR3509-3p MIMAT0016332 Arachis hypogaea miR3509-3p
AUCUAACGACUCUCAGAUAUCA
>ahy-miR3510 MIMAT0016333 Arachis hypogaea miR3510
UUAUACCAUCUUGCGAGACUGA
>ahy-miR3511-5p MIMAT0016334 Arachis hypogaea miR3511-5p
GCCAGGGCCAUGAAUGCAGA
>ahy-miR3511-3p MIMAT0016335 Arachis hypogaea miR3511-3p
UGUUACUAUGGCAUCUGGUAA
>ahy-miR3512 MIMAT0016336 Arachis hypogaea miR3512
CGCAAUAUGACAAAUAGA
>ahy-miR3513-5p MIMAT0016337 Arachis hypogaea miR3513-5p
UUAUUUCUGAGUUUGUCAUC
>ahy-miR3513-3p MIMAT0016338 Arachis hypogaea miR3513-3p
UUGAUAAGAUAGAAAUUGUAU
>ahy-miR3514-5p MIMAT0016339 Arachis hypogaea miR3514-5p
AGGAUUCUGUAUUAACGGUGGA
>ahy-miR3514-3p MIMAT0016340 Arachis hypogaea miR3514-3p
UCACCGUUAUACAGAAUCCUU
>ahy-miR3515 MIMAT0016341 Arachis hypogaea miR3515
AAUGUAGAAAUGAACGGUAU
>ahy-miR3516 MIMAT0016342 Arachis hypogaea miR3516
GCUGGGUGAUUUGACAGAAG
>ahy-miR3517 MIMAT0016343 Arachis hypogaea miR3517
CUGACCACUGUGAUCCCGGAA
>ahy-miR3518 MIMAT0016344 Arachis hypogaea miR3518
UGACCUUUGGGGAUAUUCGUG
>ahy-miR3519 MIMAT0016345 Arachis hypogaea miR3519
UCAAUCA AUGACAGCAUUUCA
>ahy-miR3520-5p MIMAT0016346 Arachis hypogaea miR3520-5p
AGGUGAUGGUGAAUAUCUUAUC
>ahy-miR3520-3p MIMAT0016347 Arachis hypogaea miR3520-3p
AAGGGAGACGUUUGAAUUAUC
>ahy-miR3521 MIMAT0016348 Arachis hypogaea miR3521
UGGUGAGUCGUAUACAUCUG

Appendix Table 7.1: Human genes determined to be affected by peanut miRNAs based on the consensus of miRanda, TargetScan, RNAhybrid and Martino et al. DEG genes.

Gene Stable ID	Gene Name	Gene Stable ID	Gene Name
ENSG00000002549	LAP3	ENSG00000138777	PPA2
ENSG00000004866	ST7	ENSG00000139832	RAB20
ENSG00000007237	GAS7	ENSG00000140464	PML
ENSG00000008735	MAPK8IP2	ENSG00000140961	OSGIN1
ENSG00000013374	NUB1	ENSG00000141576	RNF157
ENSG00000025708	TYMP	ENSG00000142089	IFITM3
ENSG00000030582	GRN	ENSG00000142347	MYO1F
ENSG00000041982	TNC	ENSG00000142949	PTPRF
ENSG00000049249	TNFRSF9	ENSG00000143226	FCGR2A
ENSG00000055332	EIF2AK2	ENSG00000143590	EFNA3
ENSG00000061918	GUCY1B1	ENSG00000144642	RBMS3
ENSG00000068001	HYAL2	ENSG00000145246	ATP10D
ENSG00000069812	HES2	ENSG00000145911	N4BP3
ENSG00000073008	PVR	ENSG00000147509	RGS20
ENSG00000075618	FSCN1	ENSG00000149131	SERPING1
ENSG00000077782	FGFR1	ENSG00000151117	TMEM86A
ENSG00000080644	CHRNA3	ENSG00000152256	PDK1
ENSG00000088305	DNMT3B	ENSG00000153234	NR4A2
ENSG00000091436	MAP3K20	ENSG00000154319	FAM167A
ENSG00000092068	SLC7A8	ENSG00000156510	HKDC1
ENSG00000099954	CECR2	ENSG00000157601	MX1
ENSG00000100342	APOL1	ENSG00000158321	AUTS2
ENSG00000100593	ISM2	ENSG00000159184	HOXB13
ENSG00000101307	SIRPB1	ENSG00000160683	CXCR5
ENSG00000102878	HSF4	ENSG00000161888	SPC24
ENSG00000104043	ATP8B4	ENSG00000163071	SPATA18
ENSG00000104679	R3HCC1	ENSG00000163993	S100P
ENSG00000105246	EBI3	ENSG00000164236	ANKRD33B
ENSG00000106003	LFNG	ENSG00000164849	GPR146
ENSG00000106952	TNFSF8	ENSG00000165071	TMEM71
ENSG00000107611	CUBN	ENSG00000165886	UBTD1
ENSG00000108255	CRYBA1	ENSG00000166432	ZMAT1
ENSG00000108679	LGALS3BP	ENSG00000166707	ZCCHC18
ENSG00000109089	CDR2L	ENSG00000167780	SOAT2
ENSG00000109906	ZBTB16	ENSG00000168491	CCDC110
ENSG00000110492	MDK	ENSG00000168961	LGALS9

ENSG00000111144	LTA4H	ENSG00000169682	SPNS1
ENSG00000111490	TBC1D30	ENSG00000170581	STAT2
ENSG00000112294	ALDH5A1	ENSG00000170955	CAVIN3
ENSG00000113319	RASGRF2	ENSG00000171877	FRMD5
ENSG00000114656	CFAP92	ENSG00000172716	SLFN11
ENSG00000115457	IGFBP2	ENSG00000173404	INSM1
ENSG00000116544	DLGAP3	ENSG00000173821	RNF213
ENSG00000117245	KIF17	ENSG00000174705	SH3PXD2B
ENSG00000117906	RCN2	ENSG00000175544	CABP4
ENSG00000119121	TRPM6	ENSG00000176697	BDNF
ENSG00000119943	PYROXD2	ENSG00000177398	UMODL1
ENSG00000121691	CAT	ENSG00000178685	PARP10
ENSG00000122224	LY9	ENSG00000181652	ATG9B
ENSG00000122986	HVCN1	ENSG00000183569	SERHL2
ENSG00000124635	H2BC11	ENSG00000183837	PNMA3
ENSG00000125084	WNT1	ENSG00000184524	CEND1
ENSG00000125730	C3	ENSG00000185634	SHC4
ENSG00000128283	CDC42EP1	ENSG00000186197	EDARADD
ENSG00000129038	LOXL1	ENSG00000186919	ZACN
ENSG00000130066	SAT1	ENSG00000187627	RGPD1
ENSG00000130653	PNPLA7	ENSG00000188404	SELL
ENSG00000131015	ULBP2	ENSG00000188886	ASTL
ENSG00000131724	IL13RA1	ENSG00000196189	SEMA4A
ENSG00000132749	TESMIN	ENSG00000196684	HSH2D
ENSG00000133069	TMCC2	ENSG00000197119	SLC25A29
ENSG00000134222	PSRC1	ENSG00000197747	S100A10
ENSG00000134508	CABLES1	ENSG00000198756	COLGALT2
ENSG00000135114	OASL	ENSG00000203943	SAMD13
ENSG00000135678	CPM	ENSG00000205683	DPF3
ENSG00000135976	ANKRD36	ENSG00000213221	DNLZ
ENSG00000136237	RAPGEF5	ENSG00000214872	SMTNL1
ENSG00000136830	NIBAN2	ENSG00000225614	ZNF469
ENSG00000137726	FXVD6	ENSG00000237190	CDKN2AIPNL
ENSG00000137959	IFI44L	ENSG00000259431	THTPA
ENSG00000138642	HERC6	ENSG00000267673	FDX2
ENSG00000138759	FRAS1	ENSG00000004468	CD38
ENSG00000139055	ERP27	ENSG00000005884	ITGA3
ENSG00000140092	FBLN5	ENSG00000008277	ADAM22
ENSG00000140876	NUDT7	ENSG00000011332	DPF1

ENSG00000141540	TTYH2	ENSG00000019144	PHLDB1
ENSG00000141738	GRB7	ENSG00000027644	INSRR
ENSG00000142178	SIK1	ENSG00000038427	VCAN
ENSG00000142794	NBPF3	ENSG00000044115	CTNNA1
ENSG00000143185	XCL2	ENSG00000053108	FSTL4
ENSG00000143375	CGN	ENSG00000060558	GNA15
ENSG00000144199	FAHD2B	ENSG00000064687	ABCA7
ENSG00000145020	AMT	ENSG00000069188	SDK2
ENSG00000145819	ARHGAP26	ENSG00000072121	ZFYVE26
ENSG00000147454	SLC25A37	ENSG00000074966	TXK
ENSG00000148600	CDHR1	ENSG00000075826	SEC31B
ENSG00000149596	JPH2	ENSG00000079215	SLC1A3
ENSG00000151892	GFRA1	ENSG00000082781	ITGB5
ENSG00000152969	JAKMIP1	ENSG00000089692	LAG3
ENSG00000153904	DDAH1	ENSG00000092009	CMA1
ENSG00000155966	AFF2	ENSG00000099219	ERMP1
ENSG00000157483	MYO1E	ENSG00000100196	KDELR3
ENSG00000158050	DUSP2	ENSG00000100427	MLC1
ENSG00000158806	NPM2	ENSG00000101210	EEF1A2
ENSG00000160191	PDE9A	ENSG00000101695	RNF125
ENSG00000161031	PGLYRP2	ENSG00000103811	CTSH
ENSG00000162739	SLAMF6	ENSG00000104413	ESRP1
ENSG00000163746	PLSCR2	ENSG00000104951	IL4I1
ENSG00000164089	ETNPPL	ENSG00000105514	RAB3D
ENSG00000164638	SLC29A4	ENSG00000106688	SLC1A1
ENSG00000165025	SYK	ENSG00000107438	PDLIM1
ENSG00000165801	ARHGEF40	ENSG00000107872	FBXL15
ENSG00000166343	MSS51	ENSG00000108551	RASD1
ENSG00000166689	PLEKHA7	ENSG00000108924	HLF
ENSG00000167114	SLC27A4	ENSG00000109684	CLNK
ENSG00000168301	KCTD6	ENSG00000110104	CCDC86
ENSG00000168890	TMEM150A	ENSG00000111012	CYP27B1
ENSG00000169604	ANTXR1	ENSG00000111331	OAS3
ENSG00000170160	CCDC144A	ENSG00000111678	C12orf57
ENSG00000170837	GPR27	ENSG00000112782	CLIC5
ENSG00000171388	APLN	ENSG00000113739	STC2
ENSG00000172349	IL16	ENSG00000115129	TP53I3
ENSG00000173156	RHOD	ENSG00000116096	SPR
ENSG00000173578	XCR1	ENSG00000116678	LEPR

ENSG00000174307	PHLDA3	ENSG00000117560	FASLG
ENSG00000175497	DPP10	ENSG00000118513	MYB
ENSG00000176293	ZNF135	ENSG00000119866	BCL11A
ENSG00000177294	FBXO39	ENSG00000120693	SMAD9
ENSG00000177842	ZNF620	ENSG00000122121	XPNPEP2
ENSG00000181004	BBS12	ENSG00000122694	GLIPR2
ENSG00000183248	PRR36	ENSG00000124507	PACSIN1
ENSG00000183780	SLC35F3	ENSG00000124785	NRN1
ENSG00000184378	ACTRT3	ENSG00000125657	TNFSF9
ENSG00000185339	TCN2	ENSG00000127507	ADGRE2
ENSG00000185915	KLHL34	ENSG00000128602	SMO
ENSG00000186652	PRG2	ENSG00000129422	MTUS1
ENSG00000187554	TLR5	ENSG00000130396	AFDN
ENSG00000188313	PLSCR1	ENSG00000130822	PNCK
ENSG00000188760	TMEM198	ENSG00000131400	NAPSA
ENSG00000196123	KIAA0895L	ENSG00000132530	XAF1
ENSG00000196569	LAMA2	ENSG00000132881	CPLANE2
ENSG00000196878	LAMB3	ENSG00000133874	RNF122
ENSG00000197506	SLC28A3	ENSG00000134323	MYCN
ENSG00000198722	UNC13B	ENSG00000134824	FADS2
ENSG00000203797	DDO	ENSG00000135541	AHI1
ENSG00000205089	CCNI2	ENSG00000135899	SP110
ENSG00000206384	COL6A6	ENSG00000136098	NEK3
ENSG00000214435	AS3MT	ENSG00000136531	SCN2A
ENSG00000222009	BTBD19	ENSG00000137507	LRRC32
ENSG00000236315	NCR3	ENSG00000137809	ITGA11
ENSG00000251369	ZNF550	ENSG00000138356	AOX1
ENSG00000262795	IFNGR2	ENSG00000138738	PRDM5
ENSG00000269743	SLC25A53	ENSG00000138792	ENPEP
ENSG00000002587	HS3ST1	ENSG00000140044	JDP2
ENSG00000005102	MEOX1	ENSG00000140479	PCSK6
ENSG00000007372	PAX6	ENSG00000141391	PRELID3A
ENSG00000010030	ETV7	ENSG00000141622	RNF165
ENSG00000015475	BID	ENSG00000142156	COL6A1
ENSG00000026025	VIM	ENSG00000142627	EPHA2
ENSG00000031081	ARHGAP31	ENSG00000143110	C1orf162
ENSG00000042286	AIFM2	ENSG00000143257	NR1I3
ENSG00000049768	FOXP3	ENSG00000143878	RHOB
ENSG00000058091	CDK14	ENSG00000144668	ITGA9

ENSG00000063176	SPHK2	ENSG00000145416	MARCHF1
ENSG00000068489	PRR11	ENSG00000147257	GPC3
ENSG00000070159	PTPN3	ENSG00000147799	ARHGAP39
ENSG00000073756	PTGS2	ENSG00000149403	GRIK4
ENSG00000075643	MOCOS	ENSG00000151364	KCTD14
ENSG00000078114	NEBL	ENSG00000152582	SPEF2
ENSG00000082014	SMARCD3	ENSG00000153443	UBALD1
ENSG00000088992	TESC	ENSG00000154447	SH3RF1
ENSG00000091656	ZFHX4	ENSG00000156587	UBE2L6
ENSG00000095932	SMIM24	ENSG00000157657	ZNF618
ENSG00000100116	GCAT	ENSG00000158477	CD1A
ENSG00000100346	CACNA1I	ENSG00000159496	RGL4
ENSG00000100600	LGMN	ENSG00000160712	IL6R
ENSG00000101353	MROH8	ENSG00000161921	CXCL16
ENSG00000103067	ESRP2	ENSG00000163357	DCST1
ENSG00000104213	PDGFRL	ENSG00000164061	BSN
ENSG00000104723	TUSC3	ENSG00000164362	TERT
ENSG00000105339	DENND3	ENSG00000164867	NOS3
ENSG00000106078	COBL	ENSG00000165272	AQP3
ENSG00000107201	DDX58	ENSG00000166086	JAM3
ENSG00000107614	TRDMT1	ENSG00000166532	RIMKLB
ENSG00000108387	SEPTIN4	ENSG00000166833	NAV2
ENSG00000108771	DHX58	ENSG00000167992	VWCE
ENSG00000109107	ALDOC	ENSG00000168542	COL3A1
ENSG00000109927	TECTA	ENSG00000169499	PLEKHA2
ENSG00000110777	POU2AF1	ENSG00000169884	WNT10B
ENSG00000111249	CUX2	ENSG00000170608	FOXA3
ENSG00000111537	IFNG	ENSG00000171105	INSR
ENSG00000112320	SOBP	ENSG00000172183	ISG20
ENSG00000113555	PCDH12	ENSG00000172794	RAB37
ENSG00000114805	PLCH1	ENSG00000173482	PTPRM
ENSG00000115594	IL1R1	ENSG00000174080	CTSF
ENSG00000116661	FBXO2	ENSG00000174807	CD248
ENSG00000117266	CDK18	ENSG00000175592	FOSL1
ENSG00000117983	MUC5B	ENSG00000177076	ACER2
ENSG00000119514	GALNT12	ENSG00000177409	SAMD9L
ENSG00000119946	CNNM1	ENSG00000179431	FJX1
ENSG00000121858	TNFSF10	ENSG00000182472	CAPN12
ENSG00000122547	EEPD1	ENSG00000183625	CCR3

ENSG00000124191	TOX2	ENSG00000184156	KCNQ3
ENSG00000124762	CDKN1A	ENSG00000184979	USP18
ENSG00000125247	TMTC4	ENSG00000185818	NAT8L
ENSG00000125895	TMEM74B	ENSG00000186350	RXRA
ENSG00000128422	KRT17	ENSG00000187033	SAMD7
ENSG00000129048	ACKR4	ENSG00000187642	PERM1
ENSG00000130270	ATP8B3	ENSG00000188672	RHCE
ENSG00000130707	ASS1	ENSG00000189058	APOD
ENSG00000131069	ACSS2	ENSG00000196369	SRGAP2B
ENSG00000132274	TRIM22	ENSG00000196724	ZNF418
ENSG00000132768	DPH2	ENSG00000197122	SRC
ENSG00000133106	EPSTI1	ENSG00000197930	ERO1A
ENSG00000134317	GRHL1	ENSG00000198794	SCAMP5
ENSG00000134575	ACP2	ENSG00000204128	C2orf72
ENSG00000135299	ANKRD6	ENSG00000205730	ITPRIPL2
ENSG00000135709	KIAA0513	ENSG00000213533	STIMATE
ENSG00000136002	ARHGEF4	ENSG00000221946	FXYD7
ENSG00000136367	ZFHX2	ENSG00000230293	MSH5
ENSG00000137198	GMPR	ENSG00000250722	SELENOP
ENSG00000137731	FXYD2	ENSG00000262446	GBA
ENSG00000138035	PNPT1	ENSG00000268043	NBPF12
ENSG00000138646	HERC5	ENSG00000004776	HSPB6
ENSG00000138764	CCNG2	ENSG00000006453	BAIAP2L1
ENSG00000139318	DUSP6	ENSG00000008282	SYPL1
ENSG00000140323	DISP2	ENSG00000012124	CD22
ENSG00000140939	NOL3	ENSG00000023902	PLEKHO1
ENSG00000141570	CBX8	ENSG00000030110	BAK1
ENSG00000141753	IGFBP4	ENSG00000039560	RAI14
ENSG00000142185	TRPM2	ENSG00000044524	EPHA3
ENSG00000142798	HSPG2	ENSG00000054356	PTPRN
ENSG00000143195	ILDR2	ENSG00000061337	LZTS1
ENSG00000143450	OAZ3	ENSG00000067955	CBFB
ENSG00000144354	CDCA7	ENSG00000069702	TGFBR3
ENSG00000145217	SLC26A1	ENSG00000072163	LIMS2
ENSG00000145850	TIMD4	ENSG00000075234	TTC38
ENSG00000147459	DOCK5	ENSG00000076864	RAP1GAP
ENSG00000148926	ADM	ENSG00000079337	RAPGEF3
ENSG00000151079	KCNA6	ENSG00000087250	MT3
ENSG00000152104	PTPN14	ENSG00000091409	ITGA6

ENSG00000153208	MERTK	ENSG00000092067	CEBPE
ENSG00000154153	RETREG1	ENSG00000099822	HCN2
ENSG00000156011	PSD3	ENSG00000100206	DMC1
ENSG00000157510	AFAP1L1	ENSG00000100453	GZMB
ENSG00000158089	GALNT14	ENSG00000101230	ISM1
ENSG00000159167	STC1	ENSG00000102003	SYP
ENSG00000160401	CFAP157	ENSG00000103855	CD276
ENSG00000161405	IKZF3	ENSG00000104419	NDRG1
ENSG00000162892	IL24	ENSG00000105227	PRX
ENSG00000163814	CDCP1	ENSG00000105668	UPK1A
ENSG00000164181	ELOVL7	ENSG00000106789	CORO2A
ENSG00000164695	CHMP4C	ENSG00000107518	ATRNL1
ENSG00000165029	ABCA1	ENSG00000108176	DNAJC12
ENSG00000165802	NSMF	ENSG00000108666	C17orf75
ENSG00000166352	IFTAP	ENSG00000109046	WSB1
ENSG00000166704	ZNF606	ENSG00000109832	DDX25
ENSG00000167131	CCDC103	ENSG00000110324	IL10RA
ENSG00000168334	XIRP1	ENSG00000111077	TNS2
ENSG00000168899	VAMP5	ENSG00000111335	OAS2
ENSG00000169607	CKAP2L	ENSG00000111961	SASH1
ENSG00000170412	GPRC5C	ENSG00000113231	PDE8B
ENSG00000170899	GSTA4	ENSG00000114455	HHLA2
ENSG00000171408	PDE7B	ENSG00000115318	LOXL3
ENSG00000172638	EFEMP2	ENSG00000116514	RNF19B
ENSG00000173193	PARP14	ENSG00000116819	TFAP2E
ENSG00000173611	SCAI	ENSG00000117594	HSD11B1
ENSG00000174697	LEP	ENSG00000118515	SGK1
ENSG00000175518	UBQLNL	ENSG00000119922	IFIT2
ENSG00000176383	B3GNT4	ENSG00000121270	ABCC11
ENSG00000177303	CASKIN2	ENSG00000122223	CD244
ENSG00000177873	ZNF619	ENSG00000122861	PLAU
ENSG00000181026	AEN	ENSG00000124615	MOCOS1
ENSG00000183486	MX2	ENSG00000124813	RUNX2
ENSG00000183785	TUBA8	ENSG00000125726	CD70
ENSG00000184500	PROS1	ENSG00000127528	KLF2
ENSG00000185499	MUC1	ENSG00000128656	CHN1
ENSG00000185924	RTN4RL1	ENSG00000129667	RHBDF2
ENSG00000186806	VSIG10L	ENSG00000130589	HELZ2
ENSG00000187583	PLEKHN1	ENSG00000130958	SLC35D2

ENSG00000188368	PRR19	ENSG00000131480	AOC2
ENSG00000188883	KLRG2	ENSG00000132698	RAB25
ENSG00000196141	SPATS2L	ENSG00000132965	ALOX5AP
ENSG00000196664	TLR7	ENSG00000134193	REG4
ENSG00000196998	WDR45	ENSG00000134326	CMPK2
ENSG00000197635	DPP4	ENSG00000135047	CTSL
ENSG00000198752	CDC42BPB	ENSG00000135625	EGR4
ENSG00000203896	LIME1	ENSG00000135925	WNT10A
ENSG00000205129	C4orf47	ENSG00000136231	IGF2BP3
ENSG00000206557	TRIM71	ENSG00000136541	ERMN
ENSG00000214686	IQCF6	ENSG00000137628	DDX60
ENSG00000223448	LTB	ENSG00000137821	LRRC49
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ENSG00000255398	HCAR3	ENSG00000138741	TRPC3
ENSG00000267041	ZNF850	ENSG00000138796	HADH
ENSG00000004399	PLXND1	ENSG00000140057	AK7
ENSG00000005513	SOX8	ENSG00000140564	FURIN
ENSG00000007866	TEAD3	ENSG00000141401	IMPA2
ENSG00000010278	CD9	ENSG00000141696	P3H4
ENSG00000017427	IGF1	ENSG00000142173	COL6A2
ENSG00000026751	SLAMF7	ENSG00000142765	SYTL1
ENSG00000033122	LRRC7	ENSG00000143184	XCL1
ENSG00000042832	TG	ENSG00000143333	RG516
ENSG00000050730	TNIP3	ENSG00000144040	SFXN5
ENSG00000059378	PARP12	ENSG00000144821	MYH15
ENSG00000064270	ATP2C2	ENSG00000145632	PLK2
ENSG00000068831	RASGRP2	ENSG00000147403	RPL10
ENSG00000071575	TRIB2	ENSG00000147852	VLDLR
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ENSG00000078900	TP73	ENSG00000152689	RASGRP3
ENSG00000082196	C1QTNF3	ENSG00000153898	MCOLN2
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ENSG00000091972	CD200	ENSG00000157107	FCHO2
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ENSG00000101000	PROCR	ENSG00000160791	CCR5
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ENSG00000104921	FCER2	ENSG00000164402	SEPTIN8
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ENSG00000106351	AGFG2	ENSG00000165475	CRYL1
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ENSG00000110881	ASIC1	ENSG00000170074	FAM153A
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ENSG00000114859	CLCN2	ENSG00000173548	SNX33
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ENSG00000116663	FBXO6	ENSG00000174938	SEZ6L2
ENSG00000117475	BLZF1	ENSG00000175899	A2M
ENSG00000118257	NRP2	ENSG00000177096	PHETA2
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ENSG00000122643	NT5C3A	ENSG00000183734	ASCL2
ENSG00000124201	ZNFX1	ENSG00000184226	PCDH9
ENSG00000124772	CPNE5	ENSG00000185215	TNFAIP2
ENSG00000125520	SLC2A4RG	ENSG00000185897	FFAR3
ENSG00000126231	PROZ	ENSG00000186480	INSIG1
ENSG00000128512	DOCK4	ENSG00000187193	MT1X
ENSG00000129195	PIMREG	ENSG00000188282	RUFY4
ENSG00000130303	BST2	ENSG00000188747	NOXA1
ENSG00000130813	SHFL	ENSG00000189221	MAOA
ENSG00000131381	RBSN	ENSG00000196557	CACNA1H
ENSG00000132357	CARD6	ENSG00000196782	MAML3
ENSG00000132837	DMGDH	ENSG00000197256	KANK2
ENSG00000133121	STARD13	ENSG00000198576	ARC
ENSG00000134321	RSAD2	ENSG00000203791	EEF1AKMT2
ENSG00000134716	CYP2J2	ENSG00000204161	TMEM273

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ENSG00000136048	DRAM1	ENSG00000221955	SLC12A8
ENSG00000136404	TM6SF1	ENSG00000232070	TMEM253
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ENSG00000137747	TMPRSS13	ENSG00000262655	SPON1
ENSG00000138119	MYOF	ENSG00000138722	MMRN1

Appendix Table 7.2: Novel miRNAs predicted by miRDeep2; provisional IDs are appointed by miRDeep2 to identify the novel miRNAs ad interim and are not scientific names.

Provisional ID	Total Read Count	Consensus Mature Sequence	Precursor Coordinate
NC_037630.1_24961	19469	uccgcaguagcacuuugg	NC_037630.1:47090141..47090207:-
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NC_037623.1_11692	10253	uccucguguugcaccucu	NC_037623.1:41011780..41011845:-
NC_037628.1_19864	7772	uguguguguuucggucucca	NC_037628.1:2671137..2671228:-
NC_037633.1_31422	5017	uugacagaagagagugagcac	NC_037633.1:148445258..148445342:+
NC_037627.1_17618	4949	uugacagaagagagugagcac	NC_037627.1:94596587..94596670:+
NC_037637.1_39664	4949	uugacagaagagagugagcac	NC_037637.1:117270419..117270502:+
NC_037620.1_5798	3529	aaccaggcucugauaccauga	NC_037620.1:132995774..132995858:-
NC_037629.1_22230	3350	ucggaccaggcucauucccc	NC_037629.1:14952880..14952966:-
NC_037619.1_2948	3350	ucggaccaggcucauucccc	NC_037619.1:11577870..11577956:-
NC_037623.1_11265	1985	uaaagcugccagcaugaucug	NC_037623.1:3867195..3867285:-
NC_037629.1_21188	1583	cuuaaucaaacucuugucauc	NC_037629.1:18992602..18992662:+
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NC_037628.1_19724	1266	ugacagaagagagugagcac	NC_037628.1:146594187..146594272:+
NC_037618.1_921	1244	ucgauaaaccucugcauccag	NC_037618.1:108768493..108768575:+
NC_037628.1_20480	1244	ucgauaaaccucugcauccag	NC_037628.1:132159862..132159944:-
NC_037633.1_32668	1158	ugacagaagagagugagcac	NC_037633.1:149879598..149879687:-
NC_037634.1_35080	1130	ugacagaagagagugagcac	NC_037634.1:133213448..133213537:-
NC_037625.1_14876	1130	ugacagaagagagugagcac	NC_037625.1:16773912..16774001:-
NC_037626.1_16946	1093	ucuucccuacaccuccauacc	NC_037626.1:112968498..112968581:-
NC_037636.1_37949	1093	ucuucccuacaccuccauacc	NC_037636.1:155462962..155463047:+
NC_037619.1_2822	1094	cuguggcuggauugaaucc	NC_037619.1:4980508..4980549:-
NC_037618.1_709	1002	uuucauuccauacaucaucua	NC_037618.1:97768187..97768269:+
NC_037628.1_20750	993	uuucauuccauacaucaucua	NC_037628.1:146528494..146528577:-
NC_037619.1_2047	403	uugauugagugacugauagcu	NC_037619.1:14681525..14681583:+
NC_037623.1_12073	341	ugacagaagaaagugagcac	NC_037623.1:111177781..111177870:-

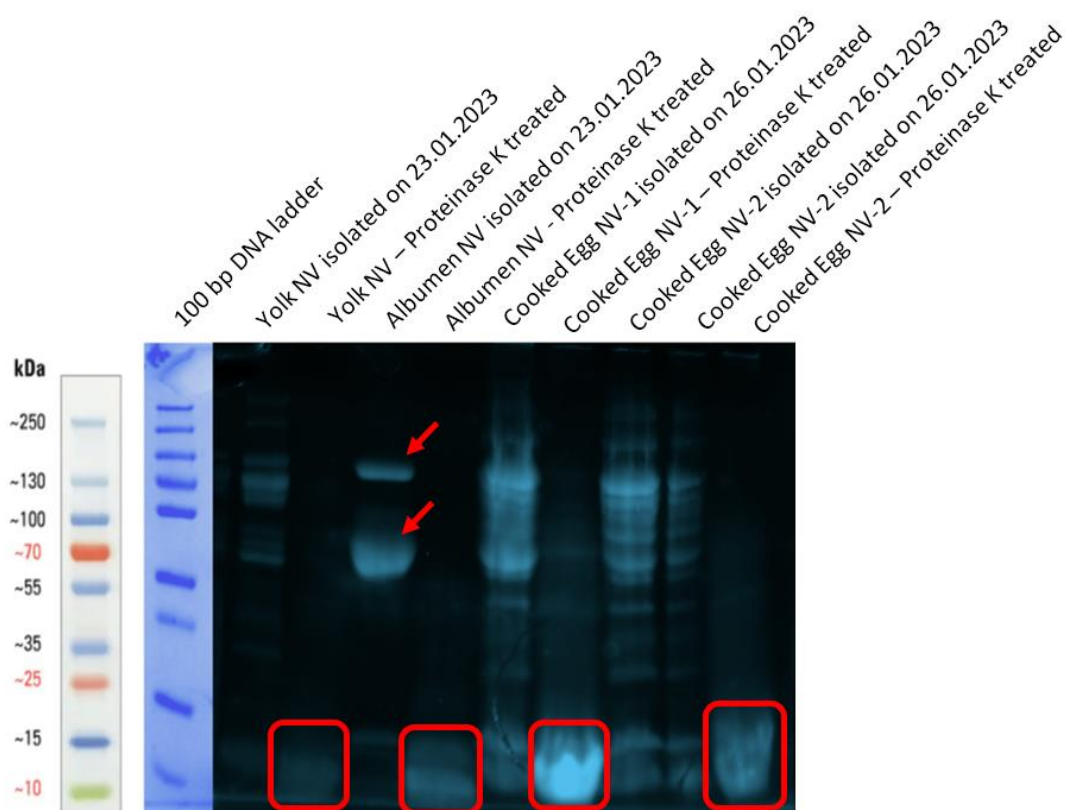
NC_037624.1_12360	203	uucugaguuuugauuauacgug	NC_037624.1:2445598..2445679:+
NC_037635.1_36886	213	gaguugaucugguagauuucggcc	NC_037635.1:128108732..128108800:-
NC_037633.1_32633	194	gugucuguauguaauucgaucac	NC_037633.1:148491535..148491622:-
NC_037634.1_34451	153	uuaucaaaaggaaucacaguug	NC_037634.1:26396458..26396542:-
NC_037624.1_13443	148	uuaucaaaaggaaucacaguug	NC_037624.1:21227048..21227130:-
NC_037619.1_2049	145	ugguaagaguuuugauuuaagu	NC_037619.1:14683767..14683826:+
NC_037633.1_31138	158	uccgcaguggcacuuugg	NC_037633.1:128383740..128383806:+
NC_037631.1_25603	118	cgcgugucucuguguuacaccgu	NC_037631.1:2463223..2463280:+
NC_037629.1_21226	80	gugucuguguguuuuucgaaucau	NC_037629.1:24853321..24853405:+
NC_037633.1_32374	88	gaggaguguguaauucauugaggu	NC_037633.1:127280477..127280564:-
NC_037631.1_26315	60	cuagauucuggugcacugacc	NC_037631.1:133572025..133572108:+
NC_037632.1_28784	54	ucagcugcaguuacuaucaugugc	NC_037632.1:158310975..158311061:+
NC_037620.1_4533	46	cauuucgauuuguguacuauuucac	NC_037620.1:131293816..131293907:+
NC_037622.1_9676	40	uucccaaaaccgccauucca	NC_037622.1:91294820..91294881:-
NC_037622.1_8038	57	gacggucugguucauguaguugac	NC_037622.1:5006650..5006699:+
NC_037632.1_27840	57	gacggucugguucauguaguugac	NC_037632.1:5006650..5006699:+
NC_037625.1_14094	29	agcauuguggaccauuaugaugagu	NC_037625.1:18436858..18436931:+
NC_037620.1_5592	32	cugcggauaugcggauccgaugug	NC_037620.1:116551347..116551414:-
NC_037618.1_379	37	acguguggccuggcgauuuccucc	NC_037618.1:39354650..39354718:+
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NC_037629.1_22756	46	uuggagaauuuuugaacgug	NC_037629.1:116311995..116312074:-
NC_037631.1_26872	24	cugagacgaauuaucaugaau	NC_037631.1:10345206..10345282:-
NC_037632.1_29882	63	uuugaacggacuagugaacuaacu	NC_037632.1:147122837..147122909:-
NC_037624.1_12764	24	cgaugacacuagcucuauaagacu	NC_037624.1:38406559..38406629:+
NC_037633.1_31763	15	gauugugagcuagaauugaaccug	NC_037633.1:6000486..6000572:-
NC_037623.1_11821	17	ccggauucugagucuaagugaaccc	NC_037623.1:87601029..87601077:-
NC_037631.1_26349	24	cuugucgaguugagggauaccgug	NC_037631.1:135918245..135918316:+
NC_037637.1_39597	21	gaauaucugucgagcuaccuuagu	NC_037637.1:106693018..106693103:+
NC_037633.1_31505	21	ggauucgggucuggagcaacuggu	NC_037633.1:151342346..151342399:+
NC_037630.1_24980	30	ucgacugagcuacaagaaaggagu	NC_037630.1:52570482..52570539:-
NC_037637.1_40197	24	ccucaaaggucgcagguucaacu	NC_037637.1:3476277..3476364:-
NC_037633.1_31795	54	cucgaauggaaaaugcugacgug	NC_037633.1:7246312..7246389:-
NC_037636.1_38814	21	uugaccagugacaugggacguuu	NC_037636.1:144471891..144471937:-
NC_037622.1_9616	50	caucagacuacaucgagcaugu	NC_037622.1:48877685..48877741:-
NC_037628.1_19657	31	cguauaaucacucaggaau	NC_037628.1:142795495..142795573:+
NC_037621.1_7409	17	uuagagcuggagauuuuaucc	NC_037621.1:99169825..99169914:-
NC_037622.1_8968	92	uuuaccagaucguuuuguuca	NC_037622.1:115136542..115136610:+
NC_037631.1_26614	15	cucagaucaacgguuaaacaagu	NC_037631.1:1001878..1001947:-
NC_037628.1_19873	58	ugaagcaagugaugacucug	NC_037628.1:2770101..2770188:-

NC_037627.1_18520	37	cugugugcucacucucuucug	NC_037627.1:94596591..94596664:-
NC_037621.1_7696	17	ggugacgauugcgacuguaauugc	NC_037621.1:125353974..125354058:-
NC_037637.1_40628	37	cugugugcucacucucuucug	NC_037637.1:117270423..117270496:-
NC_037630.1_23979	34	agugucuacuaaguguaauucugu	NC_037630.1:136424875..136424925:+
NC_037636.1_37653	3176	ucggaccaggcucauucccc	NC_037636.1:124871790..124871880:+
NC_037620.1_4587	42	uauaaguguuuuggggaacc	NC_037620.1:134399376..134399451:+
NC_037630.1_23991	42	uauaaguguuuuggggaacc	NC_037630.1:137209309..137209384:+
NC_037631.1_27494	17	ggugacgauugcgacuguaauugc	NC_037631.1:139733363..139733447:-
NC_037632.1_28162	26	agcauuguggaccuuagaugagu	NC_037632.1:31241760..31241836:+
NC_037632.1_28852	92	uuuaccagaucuguuuguuca	NC_037632.1:160191952..160192018:+
NC_037624.1_13537	21	gugcgagcgugacguguauugcu	NC_037624.1:35364885..35364936:-
NC_037619.1_2391	24	gucacuuccgggugcuuucagc	NC_037619.1:92604043..92604096:+
NC_037632.1_28895	33	uccguguguaagaauauccuac	NC_037632.1:562590..562664:-
NC_037629.1_21549	24	gucacuuccgggugcuuucagc	NC_037629.1:107430444..107430497:+
NC_037632.1_28453	221	uuuauaauugcucaaaagg	NC_037632.1:137582791..137582870:+
NC_037618.1_1247	15	uaauuguaaaccgagcuauaacgu	NC_037618.1:13919746..13919802:-
NC_037622.1_9009	33	uccguguguaagaauauccuac	NC_037622.1:562590..562664:-
NC_037635.1_35258	20	uuuauugcguuugcaccugc	NC_037635.1:5084182..5084269:+
NC_037633.1_32632	37	cugugugcucacucucuucug	NC_037633.1:148445262..148445336:-
NC_037625.1_14170	20	uuuauugcguuugcaccugc	NC_037625.1:29307986..29308073:+
NC_037621.1_6327	128	gacugcggaucggauuguaucggc	NC_037621.1:29628245..29628326:+
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NC_037633.1_30340	76	ugcuuaggaggaugugucuua	NC_037633.1:5976281..5976369:+
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NC_037633.1_31708	23	gcugaguucgugagcugaugagcc	NC_037633.1:876335..876412:-
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NC_037627.1_17559	105	guugauaucugagaccguuag	NC_037627.1:88360268..88360348:+
NC_037636.1_38336	48	uaacgacucugaacuaacaucuc	NC_037636.1:14171097..14171178:-
NC_037618.1_1724	2804	uuuggacugaaggagcucc	NC_037618.1:106891699..106891770:-
NC_037637.1_39277	2804	uuuggacugaaggagcucc	NC_037637.1:9197747..9197818:+
NC_037635.1_36557	40	uugcgaaccugaucuguagugagc	NC_037635.1:32773388..32773472:-
NC_037636.1_37504	13	guuguucaaggaccugcaaacgc	NC_037636.1:37959958..37960014:+

NC_037636.1_39003	85	uuaucaagggaucacagu	NC_037636.1:155779549..155779627:-
NC_037624.1_12794	26	agcauuguggaccuauagagagu	NC_037624.1:48492979..48493057:+
NC_037635.1_35386	17	cuuccaucuagcucgugagacuu	NC_037635.1:13160460..13160516:+
NC_037618.1_1358	300	cugcuucgaacuacauuaaacgu	NC_037618.1:47393120..47393209:-
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NC_037627.1_17687	45	guguguaauucgaaccaauu	NC_037627.1:103412395..103412469:+
NC_037625.1_15317	22	uagauaaaguugauaacugagagu	NC_037625.1:47213057..47213140:-
NC_037630.1_23540	24	augacauuacgaacuauuaacau	NC_037630.1:32222914..32222970:+
NC_037625.1_14888	42	cggacgguccgugaccugagacgu	NC_037625.1:17225810..17225876:-
NC_037630.1_24427	15	caaaugauuugguugaugugu	NC_037630.1:5685205..5685251:-
NC_037637.1_40688	47	cccagauaugaacaaaagagcau	NC_037637.1:126705110..126705157:-
NC_037625.1_14205	123	cggacgguccgauuuauacuccau	NC_037625.1:30311246..30311339:+
NC_037629.1_22618	25	aaugguccguuagguuauucug	NC_037629.1:106445212..106445259:-
NC_037625.1_15152	21	agacugugucaccuccaaacgc	NC_037625.1:37394505..37394595:-
NC_037625.1_14082	25	gacagcugcugcauugaacgaggc	NC_037625.1:17474245..17474315:+
NC_037619.1_2032	21	ucguguguaauucgaacuaauu	NC_037619.1:13042120..13042197:+
NC_037622.1_8268	25	gacagcugcugcauugaacgaggc	NC_037622.1:32427144..32427214:+
NC_037634.1_33409	15	ugcucuaacuauagaacuacau	NC_037634.1:33429071..33429156:+
NC_037635.1_36179	59	guagugugcggaucugauccgauc	NC_037635.1:25255556..2525623:-
NC_037624.1_12571	14	uuauaugaagacaacugucgug	NC_037624.1:13242408..13242490:+
NC_037635.1_35567	11	uuggagacuaauuugaugauu	NC_037635.1:34737199..34737278:+
NC_037633.1_32645	20	caauccgaucggaauacuugc	NC_037633.1:149000385..149000447:-
NW_021639479.1_41337	13	ccuagucugaacgguaacugcuc	NW_021639479.1:17253..17318:-
NC_037637.1_39647	15	uugguugacuauuugauagagu	NC_037637.1:114806025..114806085:+
NC_037631.1_26669	13	ccuagucugaacgguaacugcuc	NC_037631.1:3022423..3022488:-
NW_021639479.1_41341	13	ccuagucugaacgguaacugcuc	NW_021639479.1:23184..23249:-
NC_037630.1_25514	177	aggauccgauuuguguacuccac	NC_037630.1:145495985..145496069:-
NC_037631.1_26679	13	ccuagucugaacgguaacugcuc	NC_037631.1:3081326..3081391:-
NC_037631.1_26673	13	ccuagucugaacgguaacugcuc	NC_037631.1:3028356..3028421:-
NC_037622.1_9717	619	uccacgcucaccgcacca	NC_037622.1:94351588..94351633:-
NC_037630.1_23535	13	cgaauuguguaccugcuaugc	NC_037630.1:31782677..31782722:+
NC_037630.1_24221	77	gugugaucuucuauguugaauau	NC_037630.1:165510..165594:-
NC_037630.1_23561	11	cgaauacauuuggacacacuu	NC_037630.1:36823883..36823948:+
NC_037629.1_21937	22	aaggacggacuaaguagaaauuu	NC_037629.1:1491388..1491444:-
NC_037624.1_13190	11	uacgacagaagaaauaccuac	NC_037624.1:3913151..3913234:-
NC_037620.1_4809	17	uacugugucagaagugagucucac	NC_037620.1:143194875..143194966:+

NC_037625.1_14663	45	ggacaguccgauuugugugaggca	NC_037625.1:5850069..5850126:-
NC_037620.1_5154	48	uccgauuuguggcuggcugacac	NC_037620.1:13733266..13733342:-
NC_037631.1_25791	257	cugggacgauuuauugcaugaacau	NC_037631.1:10344907..10344985:+
NC_037628.1_20154	20	agcucugauggcauauuccgaau	NC_037628.1:27747576..27747645:-
NC_037621.1_7267	25	gacagcugcugcauugaacgaggc	NC_037621.1:36579640..36579710:-
NC_037623.1_10789	21	ugcucggauauggugaacugcu	NC_037623.1:98344002..98344065:+
NC_037618.1_1152	16	gugauacgaugcuguuuugagc	NC_037618.1:6746876..6746951:-
NC_037631.1_27342	257	cugggacgauuuauugcaugaacau	NC_037631.1:131187063..131187141:-
NC_037624.1_13738	11	uuggagacuaauuugaugauu	NC_037624.1:77002129..77002208:-
NC_037631.1_26600	50	cccagauaugaacaaaagagcau	NC_037631.1:659267..659314:-
NC_037619.1_2960	32	ugggucugcagguccgucgagc	NC_037619.1:13020505..13020564:-
NC_037624.1_12662	14	gauccagaucaugauuguuacuuu	NC_037624.1:21459905..21459977:+
NC_037619.1_1823	176	cgguccgauuuguguauucucac	NC_037619.1:869160..869253:+
NC_037634.1_33421	260	cugauuggaacuacauauaacgu	NC_037634.1:34421365..34421451:+
NC_037631.1_26167	12	ccguuccguucacagaaguccgu	NC_037631.1:123986742..123986810:+
NC_037632.1_28004	317	gugucuguguaauuacaaucac	NC_037632.1:11485273..11485354:+
NC_037628.1_20551	66	aagccucaguucgcuauagu	NC_037628.1:136985819..136985891:-
NC_037630.1_23219	11	ugcuaaccguuggaauaacgccg	NC_037630.1:8992425..8992475:+
NC_037627.1_18049	194	cugauagcuguuagaugauuugac	NC_037627.1:1887991..1888051:-
NC_037634.1_33858	73	ugguucugauauaanguuguuc	NC_037634.1:122531255..122531325:+
NC_037618.1_806	9	cgguaaccaccauagaacuug	NC_037618.1:103229011..103229086:+
NC_037626.1_17089	45	cggacaguccgauuugauggagc	NC_037626.1:118245503..118245592:-
NC_037631.1_26269	258	cugggacgauuuauugcaugaacau	NC_037631.1:131185539..131185617:+
NC_037619.1_2302	24	cuuuuguuaguagacgaugacgu	NC_037619.1:85136783..85136854:+
NC_037628.1_19089	13	gugacugugaucuuuuuggcc	NC_037628.1:16517683..16517742:+
NC_037621.1_6400	23	uagcgaccgauuacucagcaacu	NC_037621.1:75420322..75420375:+
NC_037618.1_356	10	cacgcuugugcgugcguaacucuc	NC_037618.1:35498200..35498261:+
NC_037624.1_13740	11	uuggagacuaauuugaugauu	NC_037624.1:77033251..77033331:-
NC_037637.1_40136	36	cgaaguccgauuuguguucc	NC_037637.1:1680659..1680742:-
NC_037618.1_1583	14	gagauaguugaagauggacu	NC_037618.1:101325758..101325814:-
NC_037630.1_24379	18	cuaaggacuaauucauugcagauu	NC_037630.1:4273078..4273131:-
NC_037622.1_10036	15	cgugugcuacguguuacugacau	NC_037622.1:112355168..112355242:-
NC_037621.1_6743	78	agauacgaucugauacaggacac	NC_037621.1:125786302..125786387:+
NC_037633.1_32010	28	uuugcaggucucugaauaacucag	NC_037633.1:20261342..20261400:-
NC_037634.1_34029	12	gguuaaacgggcuacuggguu	NC_037634.1:133684567..133684653:+
NC_037630.1_24079	259	cugggacgauuuauugcaugaacau	NC_037630.1:140671094..140671172:+
NC_037632.1_29926	10	guuggcugcuucgaauuaucaagu	NC_037632.1:149576358..149576431:-
NC_037632.1_28160	16	uaucgguuugauucggauggauc	NC_037632.1:30503107..30503196:+
NC_037627.1_17453	10	gguuaagaucauggcuaagcuuu	NC_037627.1:46526266..46526339:+

NC_037635.1_36973	35	uauuguagaugauagaggc	NC_037635.1:131860500..131860588:-
NC_037630.1_25062	22	agaaacgugcuuuggggaucuu	NC_037630.1:101494168..101494222:-
NC_037634.1_34938	12	uauccgugguuuagagaauaucuc	NC_037634.1:125423744..125423815:-
NC_037621.1_6122	50	gugugaucucucacuuuagaaauag	NC_037621.1:4363277..4363352:+
NC_037633.1_32381	11	cuuaucucguuuauugcuguaaaca	NC_037633.1:128995240..128995310:-
NC_037635.1_35921	13	cugaaaaacugugguuaauaacgc	NC_037635.1:131120029..131120093:+
NC_037619.1_3098	45	uguggauugaaggagcua	NC_037619.1:59812177..59812239:-
NC_037626.1_16189	13	guaaacgggcuaauccgauggguu	NC_037626.1:116085061..116085147:+
NC_037634.1_33485	21	gaucugugucucugguuauuacau	NC_037634.1:51709838..51709887:+
NC_037636.1_38566	21	ugcucggauauggugaacugcu	NC_037636.1:50042432..50042495:-
NC_037635.1_36631	12	cgaagacgaguggcuaaggguu	NC_037635.1:65304934..65305016:-
NC_037636.1_37724	11	cggauagugucgaugaauugcgu	NC_037636.1:135387711..135387776:+
NC_037621.1_7240	68	uaacuguuuauucgucggaggu	NC_037621.1:28076016..28076121:-
NC_037633.1_31135	9	cugcguuuggcucuaauuccguu	NC_037633.1:128320503..128320578:+
NC_037628.1_20131	16	agcucguaccggucuggagcgagc	NC_037628.1:22161223..22161282:-
NC_037621.1_7215	9	gguggcaauguaggccggcucg	NC_037621.1:23591063..23591153:-
NC_037630.1_23301	10	ucguguguaauucgaacuaauu	NC_037630.1:13099912..13099962:+
NC_037633.1_32241	9	cgcggguucuguccuuggggc	NC_037633.1:100307179..100307236:-
NC_037621.1_6891	27	agugugcgaucugaucugauagc	NC_037621.1:2320192..2320245:-
NC_037634.1_33372	44	ugacggugcucuggaucggga	NC_037634.1:25843139..25843199:+
NC_037620.1_4858	9	gccuaccgguuuuugaacaaau	NC_037620.1:1754019..1754111:-
NC_037627.1_17256	59	gugugucucuguguccaacgu	NC_037627.1:4749594..4749673:+
NC_037620.1_5287	22	cgugucuucuguguccaaaacgu	NC_037620.1:25792919..25792990:-
NC_037619.1_2921	11	gcaggaucauguuggauacgugu	NC_037619.1:10274203..10274253:-
NC_037620.1_3926	13	cgccucuuguauggacacugacuu	NC_037620.1:11747592..11747679:+
NC_037621.1_7221	32	ccgauugaacucuggucaaacag	NC_037621.1:25775980..25776060:-
NC_037618.1_1166	47	cccagauaugaacaaaagagcau	NC_037618.1:7806303..7806350:-
NC_037632.1_29368	21	ugcucggauauggugaacugcu	NC_037632.1:16337106..16337168:-
NC_037620.1_5288	23	cgugucuucuguguccaaaacgu	NC_037620.1:25792967..25793037:-
NC_037635.1_37053	15	uggaugacugcugaaaauaaaggc	NC_037635.1:134146751..134146820:-
NC_037619.1_2031	10	ucguguguaauucgaacuaauu	NC_037619.1:13042092..13042142:+
NC_037628.1_20684	14	uuggauucgaaauuacugaaggu	NC_037628.1:143936781..143936842:-
NC_037625.1_15017	21	gacaauggguugcauucugacgu	NC_037625.1:30708973..30709034:-



Appendix Figure 7.1: Proteinase K-treated nanovesicle SDS-PAGE results. Proteinase-K treatment was effective in removing protein contaminants from nanovesicle samples. Small amino acid fragments gather near the gel's bottom, as represented by the red boxes. Red arrows highlight two of the egg proteins with the highest proportions according to the literature. These are ovalbumin (44.5 kD) and ovotransferrin (76 kDa). According to the literature, their proportions are also suitable with the thickness of their bands (Appendix Table 7.3).

Appendix Table 7.3: Known albumen proteins, phrases in bold are the proteins that are determined to be found in our original egg nanovesicle samples according to the SDS-PAGE in the Appendix Figure 7.1.

Protein	Proportion	Molecular Weight
Ovalbumin	54%	44.5 kDa
Ovotransferrin	12%	76 kDa
Ovomucoid	11%	28 kDa
Lysozyme	3.5%	~15 kDa
Ovomucin	3.5%	~8.3 kDa

Appendix Table 7.4: Read counts determined with BLAST-plus.

miRNA Name	Read Counts (PS1; PS2)	miRNA Name	Read Counts (PS1; PS2)
ahy-miR6478	36116; 31433	ahy-miR414	79; 80
ahy-miR319	35025; 34022	ahy-miR1509	60; 49
ahy-miR159a	34858; 33874	ahy-miR2118	57; 73
ahy-miR894	10861; 13916	ahy-miR166e5p	56; 30
ahy-miR2084	5451; 3782	ahy-miR5227	43; 43
ahy-miR5021	2990; 2211	ahy-miR403	36; 5
ahy-miR1511	2338; 2504	ahy-miR5234	28; 21
ahy-miR156	1978; 1307	ahy-miR5221	27; 26
ahy-miR6300	1526; 1248	ahy-miR4376	23; 22
ahy-miR845	1510; 1488	ahy-miR399c	22; 19
ahy-miR829	1509; 1500	ahy-miR1515	22; 22
ahy-miR156f	1470; 923	ahy-miR1520	21; 16
ahy-miR166h3p	1288; 1065	ahy-miR396	21; 61
ahy-miR8175	1287; 1524	ahy-miR482a	21; 6
ahy-miR166d	1286; 1064	ahy-miR169a	19; 8
ahy-miR166f	1285; 1067	ahy-miR172	18; 14
ahy-miR165	1284; 1062	ahy-miR390	18; 23
ahy-miR157	1171; 827	ahy-miR166l	15; 0
ahy-miR167a	684; 746	ahy-miR393	15; 0
ahy-miR167c5p	683; 745	ahy-miR2111	14; 19
ahy-miR167h	680; 752	ahy-miR5499	13; 11
ahy-miR15073p	574; 657	ahy-miR530	8; 5
ahy-miR162	462; 292	ahy-miR399	7; 3
ahy-miR482d3p	358; 305	ahy-miR5225	7; 8
ahy-miR156r	311; 41	ahy-miR169b	6; 5
ahy-miR6475	305; 244	ahy-miR9666	6; 1
ahy-miR156g	302; 39	ahy-miR477	5; 3
ahy-miR482a3p	201; 305	ahy-miR164	4; 10
ahy-miR2628	157; 134	ahy-miR391	3; 5
ahy-miR5244	150; 114	ahy-miR171	2; 1
ahy-miR7696	149; 143	ahy-miR4414	2; 4
ahy-miR1088	133; 111	ahy-miR2199	1; 7
ahy-miR168	119; 76	ahy-miR397	1; 2
ahy-miR7502	102; 87	Total Counts	149 283; 140 309