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New Trends in Medicine Sciences *Volume 5*

Issue 3

CONTENTS

CLINICAL AND EXPERIMENTAL RESEARCHES ***RESEARCH ARTICLES***

REVIEWARTICLE

2024 Sep

ISSN: 2717-8161 RESEARCH ARTICLE

New Trend Med Sci 2024; 5(3):168-173. https://dergipark.org.tr/tr/pub/ntms

Can Puborectalis Muscle and Abdominal Subcutaneous Adipose Tissue Thickness Indicate Dyssynergic Defecation?

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Abstract: Chronic constipation (CC) is a common issue in primary care and gastroenterology. Defined variably by patients and clinicians, CC per Rome III criteria requires symptoms for six months, present three or more days per month for three months. Dyssynergic defecation (DD), a functional constipation type, involves the failure of pelvic floor muscles to relax during defecation. This study examines the relationship between DD, puborectalis muscle thickness, and subcutaneous adipose tissue thickness via MR defecography. After ethical approval, MR defecography images of 110 patients from Ankara Bilkent City Hospital were analyzed retrospectively. Exclusions included pelvic floor descensus, rectal mass, cystocele, rectocele, or movement artifacts. The study comprised 52 DD patients and 52 matched controls. Measurements of subcutaneous adipose tissue at L5-S1 and puborectalis muscle thickness were performed on T2-weighted images. DD patients had significantly higher abdominal subcutaneous adipose tissue and puborectalis muscle thickness than controls (p=0.021, p=0.001). No significant gender differences were noted. ROC analysis revealed cut-off values of 23 mm for adipose tissue and 4.8 mm for puborectalis muscle thickness. Positive predictive values for DD were 62% for adipose tissue >23 mm, 74% for puborectalis muscle thickness>4.8 mm, and 90% for both criteria. MR defecography is essential for diagnosing DD. This study is the first to investigate the link between DD and puborectalis muscle thickness. Increased abdominal subcutaneous adipose tissue suggests a connection between DD and obesity, possibly due to increased intra-abdominal pressure leading to higher puborectalis muscle tone. Puborectalis muscle thickness>4.8 mm and abdominal subcutaneous adipose tissue thickness>23 mm are key parameters for diagnosing DD in MR defecography. These findings underscore the importance of MR defecography in diagnosing and understanding DD, leading to more precise and individualized treatments. ©2024 NTMS.

Keywords: Puborectalis; Pelvic Floor; Defecation; Constipation; Adipose.

1. Introduction

Chronic constipation (CC) is among the most common issues in clinical practice [1]. The definition of constipation varies between patients and clinicians. To address this, an international committee has developed comprehensive criteria for the diagnosis of functional bowel dysfunction. It has been reported that to qualify

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as CC according to Rome III criteria, symptoms must have started six months prior to the aadministration and have been present for three or more days per month for three months [2]. Constipation can be divided into two main types: functional and structural. Functional constipation includes conditions such as slow transit constipation, evacuation disorders and irritable bowel syndrome with constipation. Dyssynergic defecation (DD) is a specific type of defecation disorder [3]. In 1985, Preston and Lennard-Jones identified symptoms in some constipated patients associated with a failure of the pelvic floor muscles to relax, resulting in a sustained contraction of the external anal sphincter during defecation, known as 'anismus' [4]. Over time, this condition has been referred to by various names, including anal sphincter dyssynergia, pelvic floor dyssynergia, paradoxical pelvic floor contraction and obstructive defecation.

Dyssynergic defecation is obstructive defecation due to asynchronous function of puborectalis muscle. Rectal pressure increases during straining, but at the same time pressure in the anal canal increases due to paradoxical contractions of the external anal sphincter, but faeces cannot be evacuated. Although the etiology of this disease is unknown, psychogenic factors, incorrect bowel habits and obesity have been implicated[5]. The initial step in diagnosing dyssynergic defecation (DD) is to rule out any underlying abnormalities. It is essential to remember that CC can result from insufficient fiber and fluid intake, lack of physical activity, colon cancer, medications, and various metabolic, neurological, or structural conditions. There is no one gold standard method for the diagnosis of DD. Anorectal manometry and magnetic resonance (MR) defecography are the most common modalities used to diagnose DD. However, because anorectal manometry is an invasive method, MR defecography has recently become increasingly important for diagnosis. MR defecography enables the simultaneous assessment of pelvic floor with dynamicly, and rectal evacuation. It delivers high-resolution images of pelvic floor muscles, anal sphincters and surrounding soft tissues, all without radiation. However, variations in methodology and poor interobserver agreement have limited its overall effectiveness [6].

We aim to determine the relationship between DD and puborectalis muscle thickness and subcutaneous adipose tissue thickness and whether they would be supportive parameters for diagnosing DD in MR defecography.

2. Material and Methods

2.1. Study Design

After ethics committee approval was obtained, the images of 110 patients who underwent MR defecography with a prediagnosis of constipation between March 2019 and October 2023 in the radiology clinic of Ankara Bilkent City Hospital and whose abnormalities were described in the report were retrospectively analyzed (Ethical committee number: E1-2022-2408). Nineteen patients had pelvic floor descensus, five patients had rectal mass, fifteen patients had prominent cystocele and thirteen patients had prominent rectocele were excluded from the study. Also, six patients were excluded due to intense movement artifacts. The study included fifty-two patients whose MR defecography findings were compatible with DD. Age and gender paired 52 patients who underwent MR defecography between the same dates but did not have any abnormality were selected as the control group.

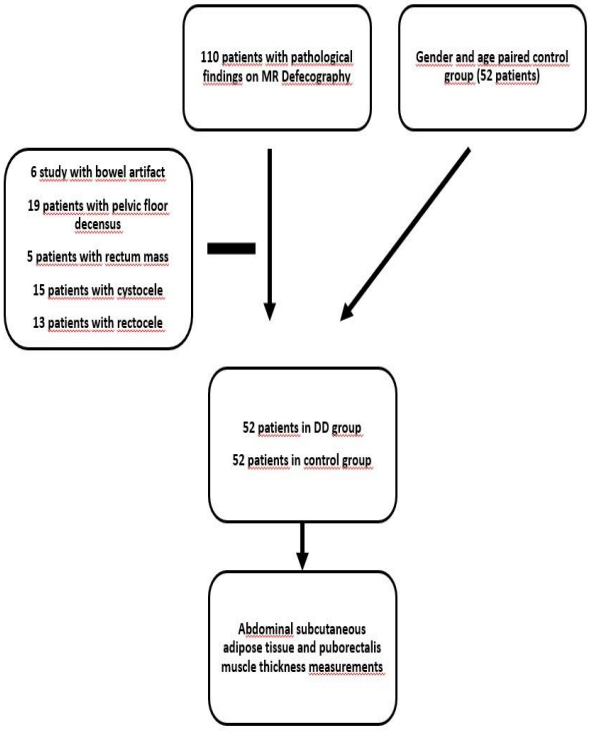

Figure 1: Flowchart of the study.

2.2. Image Acquisition

MR defecography was performed on a 1.5-T MR machine (Optima; GE Medical System, Milwaukee, Wisconsin, USA) using phased-array coils, with the patient in the supine position and knees slightly elevated on a pillow. For anatomical assessment, T2-weighted fast spin-echo images were acquired in the sagittal, axial and coronal planes at rest. Functional imaging was then performed using steady-state precession cine-type true fast imaging with steady-state precession (TrueFISP) or single-shot fast spin-echo (SSFSE) sequences in the midsagittal plane during the squeeze, stretch and defecation phases [7,8].

2.3. Image Evaluation

MR defecography images of 52 patients diagnosed with dyssynergic defecation were evaluated. To avoid measurement bias, images were anonymized and randomized prior to evaluation. Measurements were conducted by a radiologist (D.A.) with five years of experience in abdominal radiology. In both groups, the subcutaneous adipose tissue thickness at the L5-S1 intervertebral disc level was measured on T2-weighted

sagittal images. On T2-weighted axial images, the thickness of both puborectalis muscles was measured, and the mean of these measurements was recorded as "mean puborectalis muscle thickness.".

2.3. Statistical Methods

The data were processed utilizing IBM SPSS Statistics Standard Concurrent User V 26 (IBM Corp., Armonk, New York, USA). Descriptive statistics were expressed in terms of number of units (n), Mean±Standard Deviation, median (M), minimum (min), and maximum (max) values. The Shapiro-Wilk test was employed to evaluate the normality of numerical variable distributions. The Mann-Whitney U test facilitated comparisons between two categorical groups. Linear regression analysis was conducted to assess the influence of independent variables. The ROC curve analysis method was used to compare the diagnostic performance of multiple diagnostic or measurement values. The relationship between two independent categorical variables and one dependent continuous variable was analyzed using two-way analysis of variance. Pearson and Fisher exact tests were applied for comparisons of categorical variables. A p-value of less than 0.05 was considered statistically significant.

3. Results

There were 31 male and 21 female patients in either DD group and control group. The mean age in DD group was 44.92±13.3 years and in control group 42.04±12.1 years (p>0.05) (Table 1).

Table 1: Gender and age comparison of DD group and control group.

	Groups		Test Statistics
	DD group (*n*=52)	Control group (*n*=52)	*P* value
Gender, *n* (%)			
Male	21 (%40.3)	21 (%40.3)	
Female	31 (%59.7)	31 (%59.7)	>0.999
Age			
Mean±SD			
Median	44.92±13.3	42.04±12.1	>0.999
(min-max)(mm)	44 (18-73)	43 (19-70)	

Abdominal subcutaneous adipose tissue and mean puborectalis muscle thickness were significantly higher in the DD group compared to the control group (p=0.021, p=0.001) (Table 2) (Figure 2,3). There was no significant difference in abdominal subcutaneous adipose tissue and mean puborectalis muscle thickness between genders in both groups (p>0.05).

The cut-off value for abdominal subcutaneous adipose tissue thickness was 23 mm and for mean puborectalis muscle thickness 4.8 mm in ROC analysis (p<0.05). The positive predictive value for dyssynergic defecation (DD) was 62% in patients with an abdominal subcutaneous adipose tissue thickness greater than 23 mm. In patients with mean puborectalis muscle thickness greater than 4.8 mm, the positive predictive value for DD was 74%. Notably, in patients with both abdominal subcutaneous adipose tissue thickness greater than 23 mm and puborectalis muscle thickness greater than 4.8 mm, the positive predictive value increased significantly to 90%.

4. Discussion

Several findings on MR defecography have been described for the diagnosis of DD, with varying specificity and accuracy[9]. Halligan et al. demonstrated that the most common finding in patients with DD was impaired evacuation. However, they observed that impaired evacuation has low specificity and a low positive predictive value for diagnosing DD [10]. Levator ani muscle forms the primary muscular support for the pelvic floor, with the puborectalis muscle being a component of it. During defecation, the external sphincter and pelvic floor muscles relax, which enlarges the hiatus and the anorectal angle (ARA), thereby aiding the defecation process [11]. In DD, puborectalis muscle does not relax during defecation, resulting in a smaller increase or even decrease in the ARA visible on MR defecography [12]. A study of dynamic MR imaging in pediatric patients with DD found significant differences in ARA during straining and ARA change during straining between patients with DD and control group. However, they noted that abnormal ARA changes alone are not reliable for diagnosing dyssynergic defecation, as they are seen in only 50% of patients with dyssynergic defecation [13]. Also narrowing of the anorectal junction as a result of paradoxical contraction of puborectalis muscle is another finding supporting DD and is described as "sandglass-like" appearance in the literatüre [12]. Studies have yielded inconsistent findings on the occurrence of paradoxical sphincter contraction in patients with dyssynergic defecation. Reiner et al. identified a significant correlation between paradoxical sphincter contraction during straining and decreased rectal evacuation. Conversely, another study detected paradoxical sphincter contraction in both controls and patients with chronic constipation, indicating that this phenomenon is not exclusive to DD [14, 15].

Although there is no agreement on the diagnostic value of measuring the anal canal diameter using MR defecography for DD, some studies suggest that a diameter of less than 15 mm during defecation could be indicative of an incompletely relaxed anal sphincter [16, 17]. As reported in the literature, the variable sensitivity

and specificity of MR defecography findings in the diagnosis of DD remains a challenge.

To our knowledge, our study is the first to highlight the relationship between DD and puborectalis muscle thickness. Unlike other causes of chronic constipation, the primary issue in patients with DD is the excessive and paradoxical contraction of puborectalis muscle [5, 18]. Due to this, we believe that hypertrophy of puborectalis muscle, which is a voluntary muscle, develops in patients with DD.

Table 2: Comparison of abdominal subcutaneous adipose tissue and mean puborectalis muscle thickness of DD group and control group.

	Groups		Test Statistics
	DD group (*n*=52)	Control group (*n*=52)	*p* value
Abdominal subcutaneous adipose tissue thickness			
Mean±SD(mm)	30.92±12.1	21 (%40.3)	
Median(*min-max*)(mm)	31.2 (15.2-45.3)	31 (%59.7)	
			p=0.021
Mean puborectalis muscle thickness *Mean±SD(mm)*	4.90±1.55	3.84±0.932	
Median(*min-max*)(mm)	4.4 (3.38-6.68)	3.6 (2.67-4.55)	p=0.001

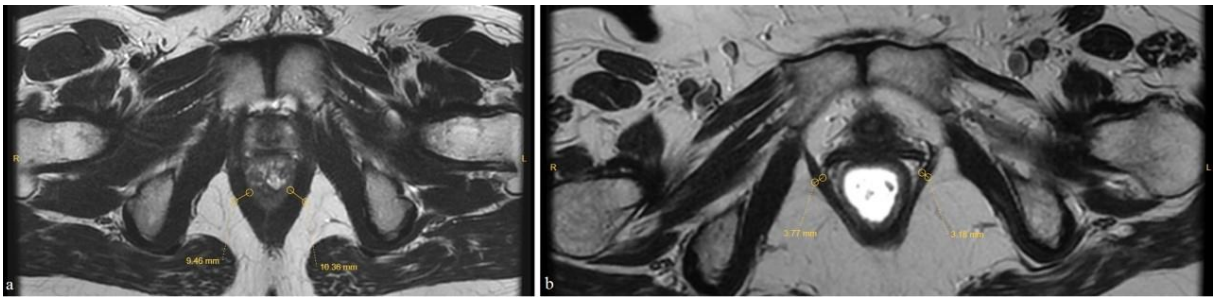

Figure 2: Mean puborectalis muscle thickness in DD group and control group (a: DD group, b: Control group).

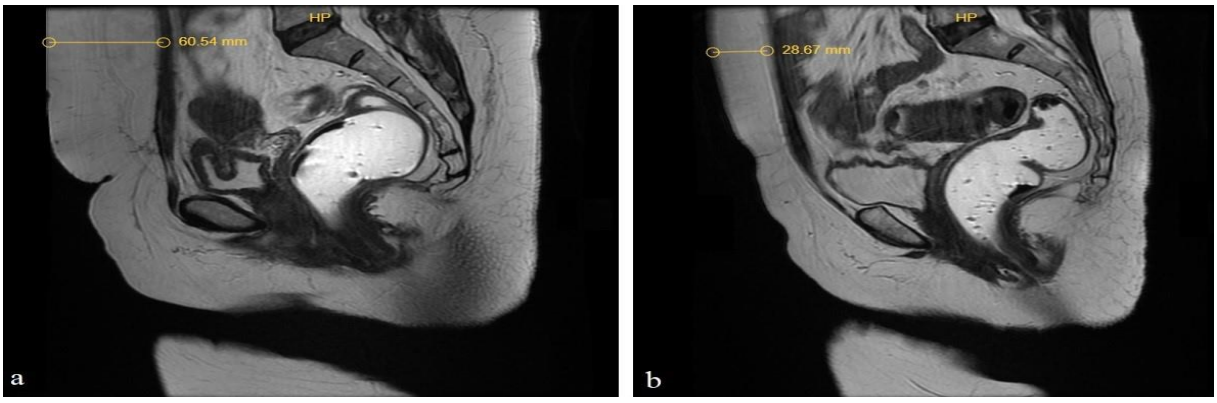

Figure 3: Abdominal subcutaneous adipose tissue thickness in DD group and control group (a: DD group, b: Control group).

Another result of our study was that abdominal subcutaneous adipose tissue thickness was higher in patients with DD. The relationship between obesity and pelvic organ disorders-prolapse (POD) remains uncertain and requires further research. While most studies suggest an association between obesity and POD, some don't show a statistically significant difference. In a study of 16.608 women, Kudish et al found that POD progression was correlated with increasing body weight. Similarly, in Wasserberg's study of 358 morbidly obese women, over 90% had pelvic floor disorders, and 50% reported that these symptoms affected their quality of life [19–21]. Although there are studies in the literature linking obesity to pelvic organ disorders, no study has investigated the relationship between obesity and DD. In our study, we observed that patients with DD had a greater thickness of abdominal subcutaneous adipose tissue, suggesting

a possible association between DD and obesity. We hypothesize that the increased abdominal subcutaneous adipose tissue leads to a continuous increase in intra-abdominal pressure, resulting in a higher tone of the puborectalis muscle to maintain continence. We also believe that the puborectalis muscle is thicker in these patients due to the increased tone. However, further studies are needed to support these hypotheses.

This study has several limitations. The relatively small number of patients and the retrospective nature of the study limit the accuracy of our results. In addition, the fact that the measurements were performed by a single radiologist may have led to measurement errors. In order to prevent this, measurements were performed after the patient images were anonymized and randomized.

5. Conclusion

In conclusion, mean puborectalis muscle thickness greater than 4.8 mm and abdominal subcutaneous adipose tissue thickness greater than 23 mm in patients with suspected DD support the diagnosis of DD.

Limitations of the Study

This study has some limitations. The relatively small number of patients and the retrospective nature of the study limit the accuracy of our results. In addition, the fact that the measurements were performed by a single radiologist may have led to measurement errors. In order to prevent this, measurements were performed after the patient images were anonymized and randomized.

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The content of the publication is entirely the authors' responsibility, and the authors examined and edited it as necessary. Each author states that the submitted article, either in full or in part, has not been previously published or is not being assessed for publication as an original article in either printed form or as digital media.

Conflict of Interests

The authors declare that there is no conflict of interest and this study was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author Contributions

Eren Çamur: Materials, Analysis and /or Interpretation, Literature Review, Writing, Critical Review.

Dilek Acar: Conception, Design, Supervision, Data Colletion and/or Processing.

Ethical Approval

The study was approved by Ankara City Hospital, Ethics Committee 1 with approval number E1-2022-2408.

Data sharing statement

All data underlying the results are available as part of the article and no additional source data are required.

Consent to participate

No consent to participate is required for this study.

Informed Statement

No informed statement is required for this sutdy.

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Enhancing Diagnostic Accuracy in Body Packing Cases: The Impact of Preliminary Diagnosis Awareness on Computed Tomography Evaluation

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Abstract: Body packing refers to the concealment of illegal substances within the body. This study aims to evaluate the computed tomography (CT) findings of body packing cases, and to assess whether considering the possibility of body packing in preliminary diagnosis will affect the accuracy of specialists evaluating in the emergency and intensive care departments. 20 body packing cases were retrospectively examined for the presence of foreign bodies. A control group was created from 20 non-contrast abdominal CT images. Re-evaluation involved four radiologists. Before evaluating, two radiologists were advised they could be body packers. In 18 (90%) of 20 body packers, foreign bodies were visible in the intestinal lumen, mostly 14 (70%) in the colon. Radiologists who were given preliminary diagnosis, correctly identified all 18 (100%) intestinal foreign body cases and did not make any false positives. Two other radiologists correctly identified 16 (88.9%) cases and missed 2 (11.1%) cases and there was significantly difference ($p<0.001$). In conclusion, packaged foreign bodies being observed most commonly in colonic segments. Evaluating without knowledge of the preliminary diagnosis of body packing significantly reduces the diagnostic accuracy. Keeping body packing cases in mind in emergency and intensive care departments in centers where they may be more prevalent can increase the diagnostic rate. When a tentative diagnosis is known, there is a greater chance of finding foreign bodies on CT scans, which increases diagnostic accuracy. This is especially true in high-prevalence settings where emergency and intensive care units may experience body packing.

Keywords: Body Packing; Computed Tomography; Pre-Diagnosis; Foreign Bodies/Diagnosis; Drug Trafficking.

1. Introduction

Body packing refers to the concealment of illegal substances, such as drugs, within the body, primarily within the gastrointestinal tract. The phenomenon of body packing was initially documented in the scientific literature in 1973 by Deitel and Syed [1]. In their study, they detailed a case involving a 21-year-old individual who experienced a partial blockage of the small intestine because of ingesting a condom stuffed with hashish. The practice of body packing has been acknowledged as a means of illicit drug transportation for a period exceeding four decades. The recent rapid growth in global travel and trade further complicates

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the challenges law enforcement faces in intercepting these substances.

Body packers utilize a deliberate method wherein pharmaceuticals are either ingested or concealed within bodily cavities such as the vagina, stomach, and ears. Body packers possess the capability to transport an approximate quantity of 1 kg of illicit substances, typically distributed across many packages. The packets are enclosed utilizing a diverse range of materials and forms, such as condoms, plastic bags, capsules, latex gloves, or balloons [2,3]. The packets commonly include of many narcotics, including heroin, cocaine, opium, cannabis, amphetamines, 3,4-methylenedioxymethamphetamine (commonly known as 'ecstasy'), marijuana, and derivatives of methamphetamine. Heroin is widely recognized for its potency, being nearly twice as strong as morphine. Body packers are colloquially known as “mules”, ‘‘internal carriers", "swallowers", "couriers" and have increasingly resorted to ingesting or inserting packets of illicit substances in an attempt to evade detection at international borders [3-5].

The most notable medical concerns associated with body packing typically manifest as indications and manifestations of drug toxicity resulting from the leakage or rupture of packets, as well as symptoms arising from the ingestion of comparatively sizable foreign objects, such as gastrointestinal blockage or perforations [6]. There has been an observed increase in the admission of bodypackers to the surgical department [7]. However, a majority of the patients have the potential to get conservative treatment [8]. Several factors have been identified as potential predictors for the necessity of surgical intervention. These factors include a history of abdominal conditions, presence of pain, occurrence of high blockage, and detection of cocaine in the urine [9,10].

The practice of body packing has gained global recognition, as evidenced by the publication of case reports in many regions including the United States, Europe, Asia, and Africa. During the period from 1993 to 2005, a total of 1250 individuals engaged in body packing were apprehended at John F Kennedy International Airport in New York [11]. However, following the terrorist attacks that occurred on September 11, 2001, there was a notable 60 percent rise in the number of arrests related to body packing [4,12,13]. The exact cause of this increase remains uncertain, as it could be attributed to heightened trafficking activities, enhanced surveillance measures, or a combination of both factors [3]. Between the years 1990 and 2001, a significant number of fatalities, around 50, occurred in the greater New York City region due to the clandestine concealment of illicit substances. The majority of these deaths were attributed to acute toxicity resulting from drug usage [14]. While initial studies indicated that a significant number of patients experienced drug toxicity due to inadequate packaging of medications, recent data indicates that instances of leakage are infrequent and the majority of patients can be effectively treated with conservative management strategies [8,15].

Historically, the detection of body packing relied heavily on non-specific clinical symptoms, physical examinations, and conventional radiography (X-ray) [16]. However, these methods have their limitations, notably the reduced sensitivity and specificity in detecting foreign bodies, especially when the packets are well concealed. The diagnostic approach to suspected cases of body packing has undergone a major paradigm shift since the introduction and widespread use of CT scanning. Abdominal CT, in particular, has emerged as a leading imaging modality due to its high resolution and ability to generate detailed cross-sectional images of the abdominal and pelvic areas, making it highly efficient in detecting concealed illicit packets [17]. Multiple studies have underscored the superiority of abdominal CT over traditional radiography in the identification and localization of ingested packets [16,18]. The ability of CT scanning to provide multiplanar reconstructions, detailed tissue contrast, and precise packet localization is unparalleled [19]. These properties not only enhance the detection rates but also guide medical interventions, especially in cases where packet rupture or obstruction is a concern.

Another compelling advantage of CT over conventional X-ray is its ability to differentiate between organic and inorganic materials. This is particularly significant as body packers often use varying materials, from latex to plastic and even animal intestines, to wrap drugs [20]. A study by Pache et al. emphasized that abdominal CT can reliably differentiate between the various packaging materials, therefore offering a decisive tool in confirming or excluding the diagnosis [21]. However, it's essential to note the ethical concerns associated with the use of CT scanning. While it presents unparalleled advantages, there is a need for discretion in its use, especially given the radiation doses involved. Guidelines and criteria must be established to ensure that CT scans are reserved for high-suspicion cases, ensuring the optimal balance between effective detection and patient safety. Although the superiority of abdominal CT over X-Ray is known, in some cases it may be difficult to distinguish a foreign body in the digestive tract from normal intestinal content [22,23].

The aim of this study is to retrospectively evaluate the abdominal CT findings of body packing cases detected at our center. This study also aims to determine whether the accuracy of experts reviewing imaging in emergency and intensive care units would be impacted by taking into account the likelihood of body packing, particularly in colon computed tomography images, in the early diagnosis.

2. Material and Methods

Ethics committee approval was obtained from the local ethics committee for this retrospective study (ethics committee no: E- 008127893.08/01-23). The study was conducted in accordance with the Declaration of

Helsinki. Due to the retrospective design of the study, obtaining informed consent was not deemed necessary by the ethics committee.

In 2022, an operation conducted by law enforcement authorities resulted in the capture of 20 foreign nationals. Abdominal CT scans were taken in our hospital to examine the captured couriers.

Images of these 20 individuals were retrospectively examined from the hospital information management system. The presence of objects that could belong to an opaque or non-opaque foreign body within the digestive tract was assessed. The localization of foreign body detection was recorded (Figure 1). The patients were followed by law enforcement officers and definitive findings were obtained regarding illegal substances in their stools. The definitive diagnosis of body packers was obtained by stool examination.

A control group was randomly formed by obtaining 20 non-contrast abdominal CT images from a similar age group, previously taken with the same CT device. While forming the control group, patients who had undergone any intestinal surgery, had any acute intestinal pathology such as ileus, perforation, volvulus, patients with firearm injuries, or penetrating abdominal injuries were excluded, and patients without intestinal pathology were included in the study.

As a result, a total of 40 non-contrast abdominal CT images were obtained, consisting of 20 body packer cases and 20 control cases. Four radiologists with years of experience, respectively 13 years, 10 years, 9 years, and 8 years, were consulted for re-evaluation. Before evaluating the 40 CT images, two radiologists with 13 and 8 years of experience were informed that these abdominal CTs taken in the emergency and intensive care departments could potentially have a preliminary diagnosis of body packers, while the other two radiologists were asked to evaluate the abdominal CT images taken in the departments without any preliminary diagnosis. Thus, two evaluation groups were formed, one with knowledge of the body packing preliminary diagnosis and one without. Each radiologist was instructed to record any positive findings.

All non-contrast abdominal CT scans were obtained using a 16-section multi-detector computed tomography machine (Siemens Somatom, Forchheim, Germany). The following technical specifications were used in the CT machine: pitch was 0.8, rotation time was 0.6 seconds, slice thickness was 1.5 mm, tube voltage was 130 kVp, automatic tube current modulation was 70 mAs.

2.1. Statistical Analysis

To summarize the data collected in the study, descriptive analyses were performed. For categorical variables, frequency tables were utilized to present the distribution of the various categories, whereas for continuous variables, such as age, the mean, standard deviation (SD), minimum (min) and maximum (max) values observed were calculated. Chi-square test was used for comparisons between groups of radiologists who were aware of the pre-diagnosis of body packing and those who were not. The interobserver variation among the first and second group of radiologists were assessed by the kappa statistic. Observer agreement was categorized by kappa values as poor (<0.20), fair (0.20-0.39), moderate (0.40-0.59), good (0.60-0.79), or excellent (>0.80) [24]. $p<0.05$ was considered statistically significant.

3. Results

The average age of the 20 individuals included in the study group of body packers (14 male, 6 female) was 34.3±5.8 years (Table 1), while the average age of the 20 normal control group individuals (14 male, 6 female) was 36.1±5.3 years.

In the study group consisting of 20 body packer cases, foreign bodies were visible in the intestinal lumen in 18 (90%) of the patients, while in 2 cases, there was evidence of disappearance and dispersion of foreign substances within the lumen. Defined foreign bodies were observed in the gastric lumen in 2 (10%) individuals and in the duodenojejunal junction in 2 (10%) individuals. In the remaining 14 (70%) individuals, foreign bodies were observed at various levels within the colonic lumen (Table 1). None of the patients in the control group had intestinal foreign bodies.

Table 1: The regions and proportions of the body packing individuals where the foreign substance was detected by abdominal computed tomography.

Age, years (Mean ± SD)	34.3 ± 5.8	
Gender (Female/Male)	6/14	
Foreign body localization	**n**	**%**
Gastric lumen	2	10
Duodenojejunal junction	2	10
Colon	14	70
Dissappear in the intestinal lumen	2	10
Total	20	100

In all 18 (100%) cases where intestinal foreign bodies were visible, the two radiologists who were initially given the preliminary diagnosis of body packing correctly identified the patients, and none of the 20 patients in the control group had any false positive diagnoses of intestinal foreign bodies.

The two radiologists who were not given the preliminary diagnosis of body packing correctly identified 16 (88.9%) of the 18 cases in which intestinal foreign bodies were visible, while they missed 2 cases. In both cases, the foreign bodies had a non-opaque appearance. They did not make any false positive diagnoses of intestinal foreign bodies in any of the 20 patients in the control group.

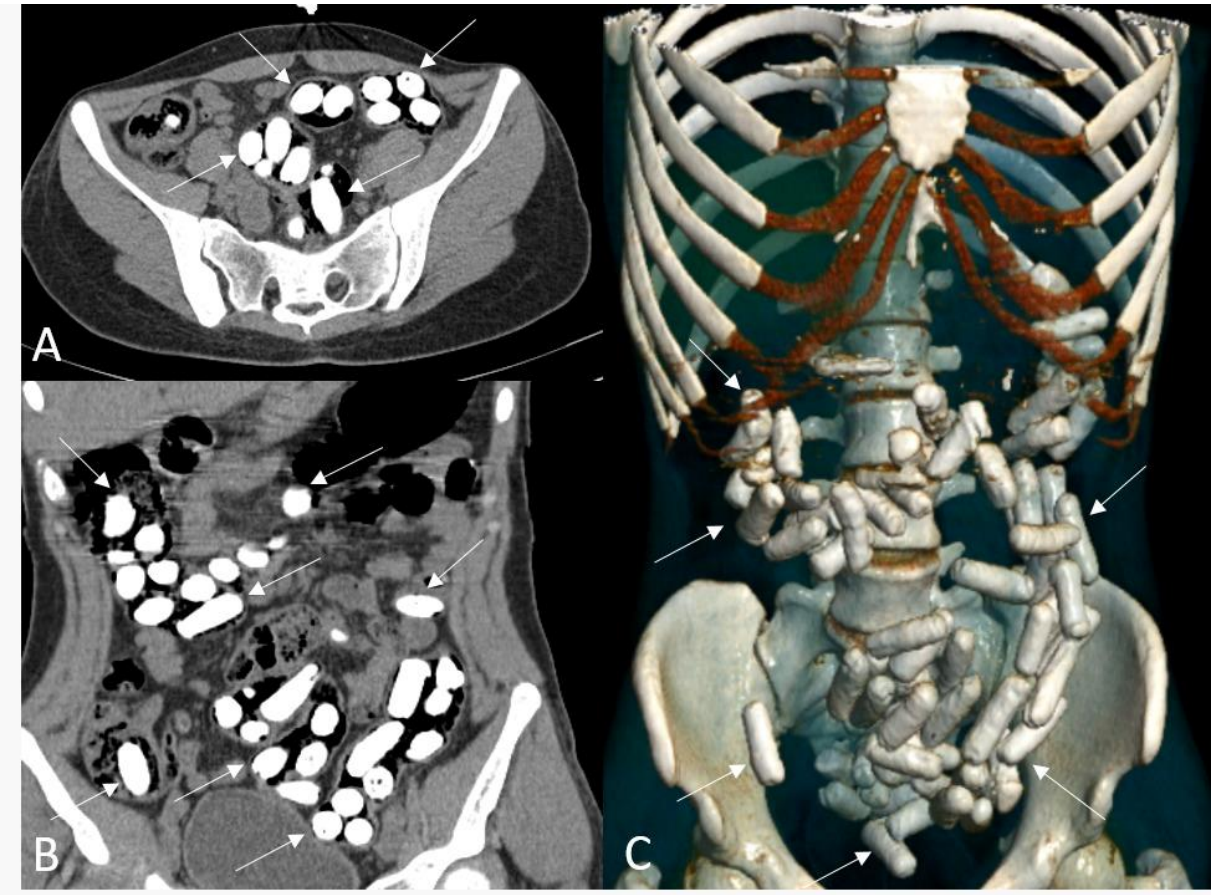

Figure 1: A 23-year-old female body packing case. A) Axial non-contrast abdominal computed tomography (CT) image shows multiple opaque foreign bodies (arrows) within the colon segments. B) Multiple opaque foreign bodies (arrows) are seen in all colon segments in coronal cross-sectional images. C) Opaque foreign bodies in the colon segments are seen on 3D reconstructive CT images (arrows).

There was excellent agreement between the two groups of radiologists with a kappa value of 0.898. However, the rate of correct diagnosis in the group given the preliminary diagnosis of body packing was statistically significantly higher than in the group not given the preliminary diagnosis ($p<0.001$).

4. Discussion

In our study, we retrospectively evaluated the CT findings of body packing cases in intensive care unit. We also created a control group without body packing and compared the diagnostic results of two different groups of radiologists, those who knew the preliminary diagnosis of body packing and those who did not. One of the most important results of our study is that it is shown that keeping the preliminary diagnosis of body packing in mind is diagnostically important. The surreptitious smuggling of illicit drugs in the body is an ongoing global challenge. These "mules", as they are colloquially known, often risk their health for the purpose of drug trafficking, putting themselves in danger of serious complications such as bowel obstruction or drug toxicity from packet rupture [3]. With the stakes so high, both from a medical and legal standpoint, accurate and swift identification of these internalized drug packets is of paramount importance. It is important for the person evaluating radiological images to have information about the patient's clinical history and existing preliminary diagnoses. In our study, radiologists who were aware of the preliminary diagnosis of body packing made accurate diagnoses with a high degree of accuracy, while the group of radiologists who evaluated without a preliminary diagnosis achieved a statistically lower accuracy rate in detecting foreign bodies. These results highlight the importance of having a preliminary diagnosis.

Law enforcement officers often detect the use of body packers and thereafter refer them to therapists for assessment, treatment, and retrieval of the concealed packets. Nevertheless, a considerable proportion of patients seek medical attention due to symptoms that are either associated with intestinal obstruction or drug toxicity. Both complications have the potential to result in fatality if the underlying disease is not identified by clinical means. None of the body packing cases in our study required surgical treatment. Foreign bodies were removed with conservative treatment and follow-up.

Despite the tendency of body packers to provide misleading information in order to evade legal consequences, it is crucial to gather a comprehensive

and precise medical history. This should encompass the specific drug being transported, the nature of the packaging material (with homemade or improvised wrapping posing a higher risk of leakage or rupture), the quantity of ingested packets, and any gastrointestinal symptoms such as pain, distention, or obstipation that may indicate obstruction or perforation. Additionally, it is important to inquire about the individual's personal use of illicit substances, as this information aids in the interpretation of toxicology testing, as well as their use of pharmaceuticals that affect gastrointestinal motility, either promoting or inhibiting it. There is a prevailing belief that the majority of those involved in body packing has precise knowledge regarding the quantity and composition of their packages, as they are obligated to provide a specified amount upon reaching their destination [25]. In our study, the 20 individuals with body packing cases were brought in for forensic examination, so there were no diagnostic challenges. However, in areas where such illegal drug trafficking is common, one of the important points emphasized by our study is the need to consider the diagnosis of body packing cases to avoid overlooking them.

The indication of a "toxic syndrome" or "toxidrome" (a collection of bodily manifestations indicating toxicity resulting from a certain medicine) implies the release of drug substance from its packaging. The opioid toxic syndrome, as observed in cases involving heroin, is characterized by symptoms including a diminished mental state, reduced respiratory function, constricted pupils, and impaired gastrointestinal motility [26]. The sympathomimetic toxic syndrome, which arises from the use of cocaine or amphetamine/amphetamine analogues, encompasses symptoms such as agitation, hypertension, tachycardia, mydriatic pupils, and diaphoresis. The presence of a significant accumulation of cocaine or amphetamine in the large intestine of a patient poses a serious risk to their life and necessitates prompt management. Due to the infrequency of trafficking including other medications such as cannabis and synthetic cannabinoid receptor agonists, it is advisable to approach patient management on a case-by-case basis. In terms of general guidelines, it is advisable to adopt a similar approach in managing patients who have swallowed substances with recognized life-threatening toxicity, such as those associated with cocaine, as well as those who have ingested medicines with similar characteristics to opioids [27].

The determination of body packing diagnosis is established using a combination of a suggestive medical history, observations made during physical examination, and the utilization of diagnostic imaging, typically involving a plain radiograph of the abdomen. For many years, the method of choice for finding these hidden packets was traditional radiography. However, because of its intrinsic benefits, abdominal computed tomography (CT) has progressively become the most widely used imaging modality in this field [28]. Traditional radiography's decreased sensitivity and specificity is one of its main drawbacks. When radiography packets are constructed of low-density materials or are positioned in a way that causes them to overlap with bones or excrement, they might cause equivocal results that can be misinterpreted [29].

On the other hand, CT's ability to provide high-resolution cross-sectional images gives it a clear edge. A study by Bulakci et al. found that abdominal CT has a sensitivity and specificity of close to 100% in detecting internalized drug packets, making it an almost foolproof technique [16]. The detailed contrast provided by CT allows for distinguishing drug packets from other abdominal contents with great precision.

Another significant advantage of CT scans is their ability to differentiate between organic and inorganic materials. As body packers use a myriad of materials to wrap drugs, ranging from latex and plastic to animal intestines, this ability is crucial [28]. With the modernization of drug trafficking techniques, traffickers are continuously innovating in packaging materials to evade detection, making the CT scan's capability even more vital. Apart from detection, CT scans provide accurate localization of the packets, which is essential for medical management. In cases where there's a concern about packet rupture or obstruction, CT imaging can guide interventions such as endoscopy or surgery [29]. Precise localization is also pivotal for forensic investigations, helping ascertain the quantity and positioning of the concealed drugs.

Despite its advantages, the use of CT scanning isn't without its controversies. Given the radiation doses involved, there's a need for discretion in its use. There is also an ethical concern regarding the involuntary examination of suspected individuals without their consent [29]. Thus, it is imperative to have clear guidelines and criteria ensuring that CT scans are reserved for high-suspicion cases, balancing effective detection and ethical considerations.

Another discussion point revolves around the cost. CT scans are notably more expensive than traditional X-rays. While they offer unparalleled precision, the economic implications, especially in lower-resource settings, cannot be ignored. However, one could argue that the costs associated with medical complications (should a packet rupture or cause obstruction) or legal implications (if drug packets go undetected) might outweigh the initial cost of the CT scan [30].

With the continuous advancement in imaging technologies, the future might bring even more efficient and safer methods for the detection of body packing. Innovations such as lower radiation dose CT protocols or the use of MRI, which does not involve ionizing radiation, might offer promising alternatives [31].

5. Conclusion

In conclusion, in body packing cases, packaged foreign bodies being observed most commonly in colonic segments, as well as in intestinal segments such as the stomach and duodenum on CT. Evaluating without

knowledge of the preliminary diagnosis of body packing significantly reduces the diagnostic accuracy. Keeping body packing cases in mind in emergency snd intensive care departments in centers where they may be more prevalent can increase the diagnostic rate. As the battle against drug trafficking continues, the medical community, in collaboration with law enforcement, will continue to rely heavily on advanced imaging techniques to protect individual and public health.

Limitations of the Study

There are some limitations to the study. The most significant limitation is that all body packing cases were apprehended by law enforcement authorities in a single event. This may suggest that every individual used a similar or the same method, and it prevented us from assessing the radiological detectability of different methods used for packaging foreign bodies. The small number of patients and the retrospective design of the study and the bias that may be caused by the small number of patients are other limitations of the study. Additionally, the radiologists who performed the evaluation were not experts in forensic medicine. Another limitation is that, despite our efforts to exclude intestinal pathologies when selecting the control group, there is a possibility of bias in the control group selection. In addition, in daily practice, feces content can be observed in various forms, and evaluating this with a limited control group of 20 individuals is another limitation of the study.

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Conflict of Interests

The authors declare that they have no conflict of interest to disclose.

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Author Contributions

Conception, HGT, BE, OCA. and SA; Design, OCA.; Supervision, OCA. and SA; Materials SA; Data Collection and Processing, TC, BE, OCA., and SA; Analysis and Interpretation, HGT, TC and SA; Literature Review, OCA. and SA; Writing, HGT, BE, OCA., and SA; Critical Review, HGT, TC, and BE.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki of 1975 (as revised in 2013), and the protocol was reviewed and approved by the Institutional Review Board (or Ethics Committee) of Erzincan Binali Yıldırım University (Date: 13 July 2023, Ebyu-kaek-no E-1537893901-000187890124467/07-2023)

Data sharing statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent to participate

None.

Informed Statement

Due to the retrospective nature of the study informed consent was not required.

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From Lab to Clinic: The Potential of Nanobubble Ozone Stored in Liposome with Pantothenic Acid (NOSLIP) in Treating Vaginal Infections with Long-lasting Effectiveness

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Abstract: In our study, pantothenic acid nanoparticle liposomal ozone solution (NOSLIP) with patent application number PCT/TR2022/050177 was used and to show that the solution maintains its effectiveness for a long time, it is developed as an antibacterial and antifungal agent and can be used for vaginal antisepsis, and it is a suitable drug that can be used for the treatment of vaginitis in the future. The antibacterial tests of NOSLIP, which was developed with a new technique, with the CLSI M07 A9 standard test method, and its antifungal activity with CLSI M27-A3 were studied. The stability test of the NOSLIP solution was kept at 55 oC for 74 days, corresponding to 2-year stability, according to the ASTM F 1980 standard. The product's particle was determined as 363nm. No growth was observed after 24-hour hemodynamic incubation with Streptecoccus agalactia (ATCC13813), E. coli (ATCC25922) bacterial suspensions adjusted to 0.5 MacFarland value and Broth medium. Again for Candida albicans (ATCC 10231), in the time-dependent efficacy test performed with a concentration of 1600 ppm, a 90% reduction in 24-hour plaque and no growth was observed at the 48th hour. In terms of effectiveness, the solution was still found to be effective after 2 years according to the ASTM F 1980 standard. It is thought that NOSLIP can be used for vaginal antisepsis with solutions to be prepared in appropriate doses due to its natural and slow release, prevent bacteria and fungi from settling on the mucosal membranes. ©2024 NTMS.

Keywords: Nanobubble Ozone; Nanoliposome; Vajinitis; Candida Albicans; Antibacterial Agent.

1. Introduction

Every year, 5-10 million women apply to various centers for sexually transmitted diseases due to infectious vaginitis [1]. The three most notable causes of infectious vaginitis are bacterial vaginosis (BV), trichomoniasis, and vulvovaginal candidiasis (VVC). BV and VVC, which are endogenous genital infections, are the agents most responsible for the etiology of vaginal discharge [2]. The most common symptoms of infectious vaginitis are vaginal discharge, itching, and a burning sensation. However, some cases are asymptomatic and are untreated [3].

Group B streptococci (Streptococcus agalactiae; GBS) are gram-positive encapsulated bacteria that can colonize the intestinal and vaginal flora in 10-30% of healthy adults [4]. *Streptococcus agalactiae* causes serious infections such as meningitis, sepsis, skin and soft tissue infections, pneumonia, urinary tract infections, and postpartum endometritis in newborns,

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pregnant women and adults with underlying diseases [4-6].

Ozone is a reactive oxygen species consisting of three oxygen atoms produced by ultraviolet light and high-pressure diatomic oxygen, and is recognized as a strong oxidative antimicrobial agent. Ozone therapy has received increasing attention in recent years and is widely known for its positive effects on infection, reperfusion injury, cancer, and dental caries [7-8]. Currently, ozone therapy is a new concept in the clinical treatment of vaginitis. The medical integrated ozone therapeutic apparatus uses an ozone generator to prepare a certain concentration of ozone and mixes it with filtered tap water to form ozonated water. Ozone and active molecules are in a liquid state and play a role in the sterilization of the vagina [9].

2. Material and Methods

2.1 *Solutions Preparations*

The nanobubble ozone stored in a liposome solution (NOSLIP), which was prepared with a method that is different from standard ozonation mechanisms, is protected by patent PCT/TR2022/050177. While preparing the solution, pantothenic acid (vitamin B5) was attached to the carrier nanomolecules to support the vaginal mucosa. The antibacterial, antiviral, biocompatibility and cytotoxicity tests of the NOSLIP solution before it was decorated with panthotenic acid were studied and published [12].

2.2 *Characterization of NOSLIP*

Size polydispersity (PDI), zeta potential, hydrodynamic diameter (Z-average size), dynamic Light Scattering (DLS) measurements were taken at 20 °C from three independent samples with a Zetasizer Nano ZS instrument (Malvern Instruments Ltd., UK) containing a solid-state HeNe laser (λ=633nm) at a scattering angle of 173°.

2.3. *In vitro Anti-Fungal Activity of the NOSLIP Solution*

According to CLSI M27-A3 [11] recommendations, antifungal drugs were diluted in an RPMI 1640 medium containing 0.2% glucose and were distributed at the appropriate concentration onto U-bottom microdilution plates. The inoculum suspension was adjusted to a final concentration of 0.5 x 10^3-2.5 x 10^3 cells/ml and it was dispensed into microdilution wells with different antifungal concentrations. Plates were incubated at 35 °C. While determining the MIC value for Candida species according to the CLSI standard, the concentration at which a 50% decrease was observed at the end of the 24th hour from the prepared dilutions was considered to be the MIC (minimal inhibitory concentration) value. In this study, it was determined that the MIC value was 1600 ppm by performing the standard study with 3200 ppm, 2400 ppm, 1600 ppm, and 800 ppm concentrations (Table 1).

Table 1: MIC values against Candida albicans (ATCC 10231) according to the CLSI M27-A3 method.

Sample	Tube	Dilution	ppm	*Candida albicans*
NOSLIP Solution	1	1	3200	-
	2	1/3	2400	-
	3	1/2	1600	-
	4	1/4	800	+

3. Results

3.1. The NOSLIP Solution Characterization

The NOSLIP solution dimensions ranged between 48 nanometers and 2 microns. Most of the particles were found to be concentrated at 4844 and 106,1 nanometers (Figure 1).

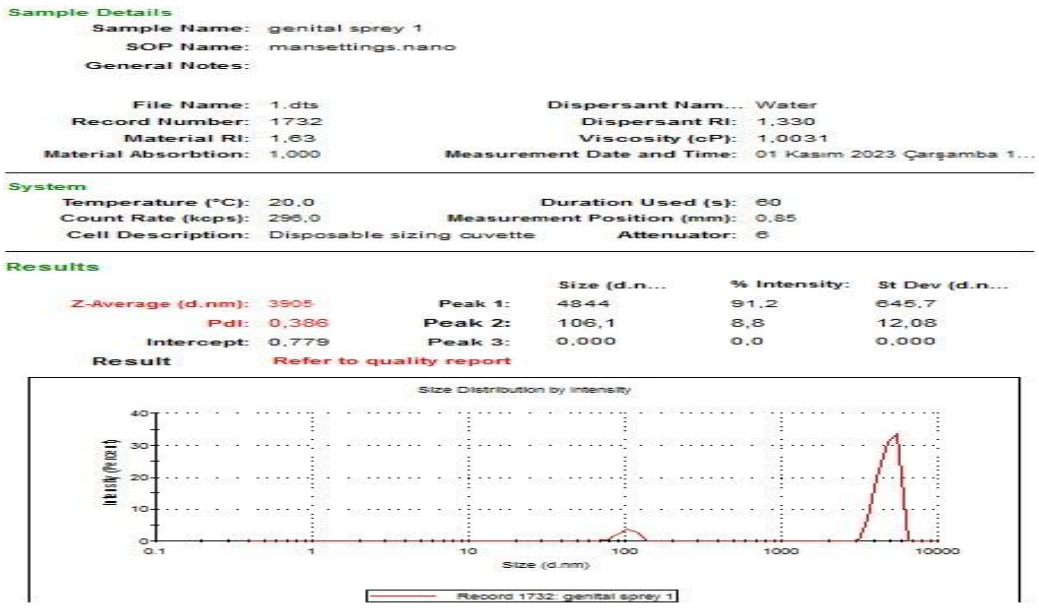

Figure 1: The Zeta size and poly dispersity index of the NOSLIP solution.

The NOSLIP solution was imaged for the first time by Scanning Electron Microscopy (SEM) and it was determined that the product was a nanomolecule (Figure 2).

3.2. Analysis of time-dependent Antibacterial Effects of the NOSLIP Solution

The MIC of the nanobubble liposomal ozone solution was determined using the CLSI M07 A9 (Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard) standard test method for Methicillin-resistant *Staphylococcus aureus* (ATCC 12493), *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25922). The ATCC25923 strains were calculated as 1562 ppm. To evaluate the time-dependent effects of the solution, the MIC value was above 1600 ppm. No growth was observed as a result of the 24-hour 37 ˚C hemodynamic incubation with *Streptococcus agalactia* (ATCC13813) and *Escherichia coli* (ATCC25922) bacterial suspensions, and Broth, a medium which was adjusted to a 0.5 MacFarland value (Table 2).

Table 2: Tests of Streptococcus agalactia (ATCC13813) and Escherichia coli (ATCC 25922) bacteria at different ppm levels nanoparticle liposomes at different times.

Time	*Streptococcus agalactia (ATCC13813)*	*Escherichia coli (ATCC25922)*
2 min.	+	+
10 min.	+	+
30 min.	+	+
1 h.	Reduction	Reduction
2 h.	-	-
3 h.	-	-
4 h.	-	-
5 h.	-	-
6 h.	-	-
24 h.	-	-

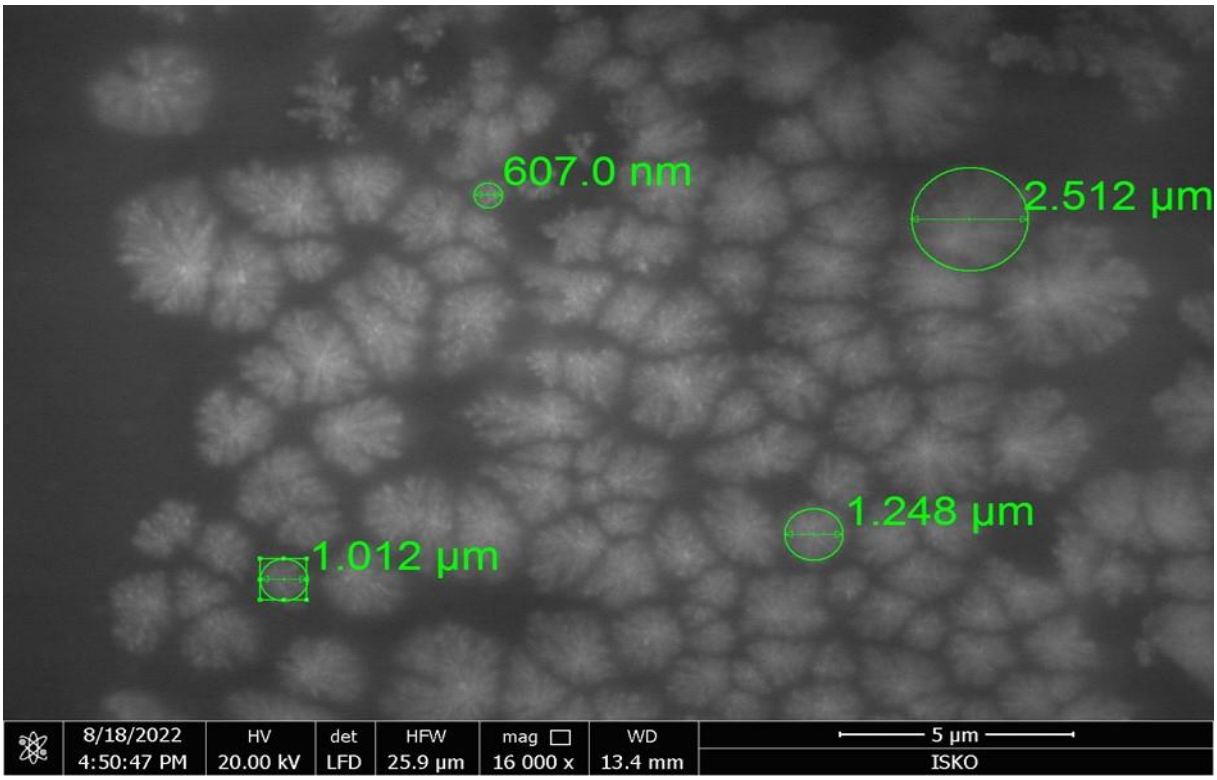

Figure 2: SEM image of NOSLIP Solution at 16 000 magnification.

3.3. Analysis of time-dependent Antifungal Effects of the NOSLIP Solution

For Candida albicans (ATCC 10231), in the time-dependent efficacy test performed with a concentration of 1600 ppm, a 90% reduction in 24-hour plaque and no growth at 48 hours were observed (Figure 3a-b).

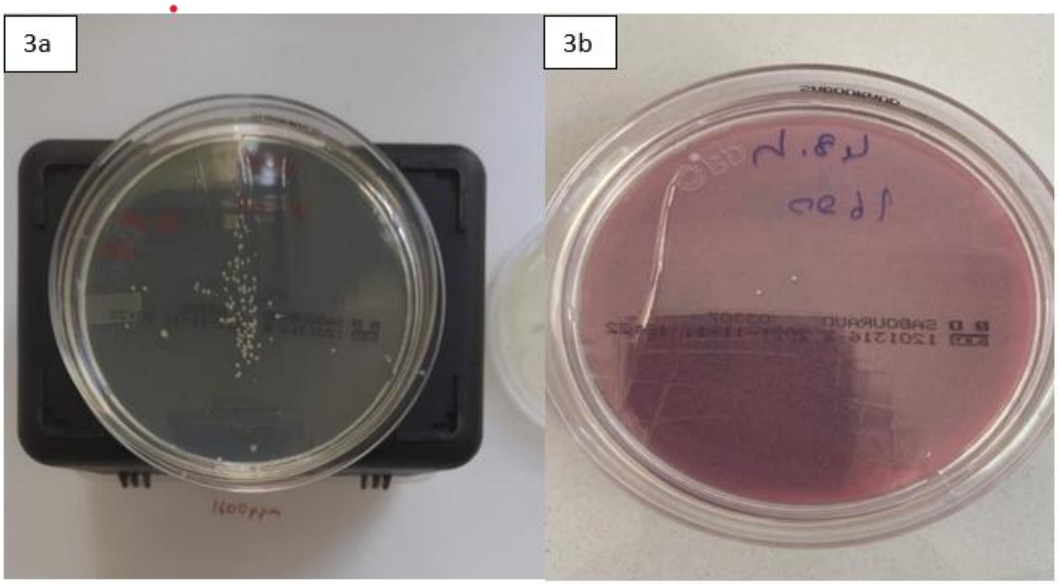

Figure 3: 3a,3b- The time-dependent efficacy test.

3.5 *A Stability Test of the NOSLIP Solution*

The ASTM F1980 (standard guide for accelerated aging of sterile barrier systems for medical devices) was used as a reference to prepare the ozone solutions in their active concentrations, and these solutions were stored at 55 °C for 74 days to determine their stability after two years. After the years, *Staphylococcus aureus* (ATCC 25922), Methicillin-resistant *Staphylococcus aureus* (ATCC 12493) and *Escherichia coli* (ATCC 25922) suspensions regulated to 0.5 McFarland turbidity were readded to the solutions. As before, the samples were obtained from the solutions at 2 min, 10 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and finally, 6 h (Table 3). The blood agar medium (Germany-Becton Dickinson) was used for cultivation of the samples and they were incubated for 24 hours at 37 °C. The presence of bacterial growth was assessed on the plates after the incubation period. The stability was defined as the preserved effectiveness of the solution during the contact period, at the concentration where the antibacterial activity was previously recorded.

Table 3: ASTM F 1980 Stored at 55°C for 74 Days NOSLIP Solution.

Time	*Streptococcus agalactia (ATCC13813)*	*Escherichia coli (ATCC25922)*
2 min.	+	+
10 min.	+	+
30 min.	+	+
1 h.	Reduction	Reduction
2 h.	-	-
3 h.	-	-
4 h.	-	-
5 h.	-	-
6 h.	-	-
24 h.	-	-

The products and results used in our study are available to Data Availability.

4. Discussion

The exploration of NOSLIP as a potential treatment for vaginitis presents a promising avenue for addressing both the pathogenic and ecological aspects of vaginal health. Traditional treatments often focus solely on eradicating pathogens, which can inadvertently disrupt the delicate balance of the vaginal microbiome. This disruption can lead to further complications, including recurrent infections, as evidenced by the high rates of recurrence associated with bacterial vaginosis (BV) treatments that do not restore the normal flora [13] NOSLIP, with its dual action of pathogen elimination and preservation of beneficial bacteria, could represent a significant advancement in the management of vaginitis.

The vaginal microbiome is predominantly composed of Lactobacillus species, which play a crucial role in maintaining a healthy vaginal environment by producing lactic acid and other metabolites that inhibit pathogenic growth [14-15]. The introduction of NOSLIP, which utilizes ozone and pantothenic acid in a slow-release formulation, could enhance the proliferation of these beneficial bacteria while simultaneously reducing the concentration of harmful pathogens16. This aligns with findings that suggest treatments promoting Lactobacillus growth can significantly improve vaginal health and reduce the incidence of infections [17-18].

Moreover, the stability of NOSLIP solutions for at least two years, in contrast to the short half-life of ozone in water, suggests a sustained therapeutic effect that could be beneficial in clinical settings [20]. This prolonged efficacy is critical, as many existing treatments require frequent application, which can be burdensome for patients and may lead to inconsistent outcomes. The slow-release mechanism of NOSLIP not only ensures a continuous antimicrobial effect but also supports the

recovery of the vaginal microecology, which is essential for long-term health [20-21].

Clinical evidence supporting the efficacy of NOSLIP in treating vaginitis is still limited, necessitating further studies to establish its role within the broader context of vaginal health management. Previous studies have indicated that restoring the vaginal microbiome can significantly alleviate symptoms associated with vaginitis and reduce inflammatory responses [22]. For instance, the use of prebiotics and probiotics has shown promise in promoting the growth of Lactobacillus, thereby enhancing the natural defenses of the vagina against infections [23-24].

Conclusion

The potential of NOSLIP as a treatment for vaginitis lies in its ability to address both the immediate symptoms of infection and the underlying microbial imbalances. By fostering a healthy vaginal environment, NOSLIP could not only alleviate discomfort but also reduce the risk of recurrent infections, thereby improving the overall quality of life for affected individuals. Future clinical trials will be essential to validate these findings and explore the full therapeutic potential of NOSLIP in the context of vaginal health.

Limitations of the Study

In the study, evaluations were made based on in vitro experiments.

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Conflict of Interests

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None.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent to participate

None.

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None.

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Detecting The Presence of Anti-HLA Antibodies in Autoimmune Diseases

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Abstract: By creating antibodies, the immune system protects the body from foreign antigens. The immune system may occasionally sustain damage that results in a loss of tolerance to its antigens, which triggers the creation of antibodies directed against those antigens. Another challenge in solid organ transplantation is the existence of these anti-HLA antibodies. Our goal was to find out if common autoimmune disorders such type 1 diabetes (T1D), Behçet's disease (BD), and ankylosing spondylitis (AS) result in the development of anti-HLA antibodies. 100 patients with AS, 100 patients with BD, 60 patients with T1D, and 100 healthy people were included in this study. PRA screening tests were performed on serum from blood samples taken from both patients and healthy individuals to look for the presence of anti-HLA antibodies. Of the AS patients, 5 were positive for PRA class II alone, 7 were positive for both PRA class I and class II, and 1 patient was positive for PRA class I. In BD, 3 patients was positive for both PRA class I and II, 7 patients was positive for PRA class II alone, and 1 patient was positive for PRA class I. In T1D, 1 patient were positive for PRA class I, 3 patients were positive for PRA class II alone, and 2 patients were positive for both. When three patient groups were compared with the control group, there was no statistically significant difference in the detection of anti-HLA antibodies between the patient groups and the control group. ©2024 NTMS.

Keywords: Ankylosing Spondylitis, Behçet's Disease; Type 1 Diabetes, Transplantation, PRA.

1. Introduction

It is believed that hereditary and environmental factors combine to cause autoimmune disorders. According to the hereditary foundation of these illnesses, those who have specific genetic variations are more likely to develop autoimmune diseases. A major element of this genetic tendency is Human Leukocyte Antigens (HLA). HLA genes are important for immune system regulation and have an impact on the onset of autoimmune disorders. Certain HLA alleles, for instance, have been linked strongly to the onset of autoimmune disorders, including Rheumatoid Arthritis (RA), Systemic Lupus Erythematosus (SLE), and Type1Diabetes (T1D) [1].

Autoimmune disorders demonstrate that the host's immune system can generate HLA antibodies against both foreign and internal HLA antigens. These anti-HLA antibodies can be found using a test known as Panel Reactive Antibody (PRA). When patients are waiting for an organ transplant, the PRA test is frequently done to find out if they have anti-HLA antibodies. Because the largest barrier to organ transplantation is the existence of anti-HLA antibodies. Nowadays, there is also questioning and scrutiny surrounding the occurrence of anti-HLA antibodies in liver and bone marrow transplants [2, 3].

Ankylosing spondylitis (AS), a subset of spondyloarthritis that is often referred to as Bechterew's disease, is characterized by aberrant bone remodeling and inflammation in the sacroiliac joints and spine. Even though the exact cause of AS is unknown, the genetic marker HLA-B27 is strongly linked to the condition. More than 90% of AS patients have the HLA-B27 gene, according to research [4].

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A systemic inflammatory illness, Behçet's disease (BD) is primarily characterized by painful oral ulcers that recur often. Other clinical symptoms that may be present include vaginal ulcers, erythema nodosum, or acneiform pustular lesions [5]. A genetic correlation was found in 1982 between BD and HLA-B51 [6].

An autoimmune condition known as type 1 diabetes (T1D) is typified by autoantibodies destroying beta cells in the pancreas known as Langerhans islets. According to Derrou et al. (2021) the death of cells results in an insulin deficit and chronic hyperglycemia. Geographic variations in the prevalence of T1D are caused by both genetic and environmental factors. The risk of having T1D is much higher in people who carry the HLA DR4-DQ8 and HLA DR3-DQ2 alleles [7].

Studies have shown that autoimmune disorders such systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are associated with elevated levels of anti-HLA antibodies [8, 9]. Finding out if AS, BD, and T1D illnesses result in the production of anti-HLA antibodies was our goal. To do this, antibody presence was determined using the standard Luminex PRA method, which is frequently used to identify the presence of antibodies in patients with chronic kidney disease (CKD) and organ transplantation.

2. Material and Methods

In addition to 100 healthy people without any chronic illnesses serving as the control group, the study comprised 100 patients with ankylosing spondylitis, 100 patients with Behçet's disease, and 60 patients with type 1 diabetes. The study comprised patients who tested positive for HLA-B51 in Behcet's patients and HLA-B27 in AS patients. Additionally, consent forms from people aged 18 to 59 were used to choose all included patients and healthy volunteers. The tissue typing laboratory of the Atatürk University Research Hospital served as the study's location.

2.1. Sera Collection

Using a Nüve NF 048 centrifuge, blood samples taken from patients and healthy individuals were centrifuged at 4100 RPM for five minutes in order to obtain serum samples. The top part was moved into Eppendorf tubes. The acquired serum samples were kept in the proper storage environments.

2.2. Luminex PRA Test

The Luminex PRA TestThe IMMUCOR LIFECODES LifeScreen Deluxe kit was used to conduct the PRA screening test. Initially, 300 microliters of purified water were introduced into every well to moisten the filters beneath the filtered plate, and then the mixture was allowed to sit for five minutes. After that, each well was filled with 40 µl of wash buffer, 12 µl of patient serum, and 5 µl of LifeScreen Deluxe Beads from the kit. The wells were then left in the dark for 30 minutes. Following incubation, the wells were twice washed with 250 µl and 100 µl of wash buffer. After that, 50 µl of the conjugate solution that had already been made was added, and the mixture was once more incubated for 30 minutes in the dark. Following the incubation period, the wells were twice cleaned using 250 µl and 100 µl of wash buffer. After that, 30 minutes of dark incubation were spent again after adding 50 µl of the conjugate solution that had already been made. Following the incubation period, 130 µl of wash buffer was introduced into each well, and the Luminex Labscan 200 instrument was utilized for analysis. The Matchit Antibody program was used to examine the data, and MFI (Median Fluorescence Intensity) values larger than 1000 were regarded as positive.

2.3. Statistical Analyses

The mean, standard deviation, percentage, and count of the data were displayed. In 2x2 tables, the Pearson chi-square test was utilized to compare categorical variables if the expected value was below 20% and (>5), the Yates chi-square test was employed to compare values below 20% and (3-5), and the Fisher's Exact test was utilized to compare values if the expected value was (<3). When comparing categorical variables in tables bigger than 2x2, the Fisher-Freeman-Halton test was utilized when the expected value was less than 5, and the Pearson chi-square test was employed when the expected value was greater than 5. P-values were regarded as statistically significant if they were less than 0.05. One-way ANOVA was used to statistically analyze the MFI readings in the PRA screening test.

3. Results

There were 100 volunteers in the control group, 59 of them were female and 41 were male. The average age of the male population was found to be 39.17±10.3 years, and the female population was found to be 30.42±8.94 years. Three female volunteers tested positive for PRA class I, one positive for PRA class II, and one positive for both PRA class I and II, per the results of the PRA screening. Out of the male subjects, one tested positive for PRA class I and two tested positive for PRA class II. Within the control group, statistical analysis showed no discernible difference between male and female volunteers (Table 1).

Table 1: PRA results and mean ages of the control group.

	Female (n=59)	Male (n=41)	p
Number of participants	59	41	-
Mean Age (Years)	30.42±8.94	39.17±10.3	-
Positive PRA Class I	3 (5.08%)	1 (2.43%)	0.642
Positive PRA Class II	1 (1.69%)	2 (4.87%)	0.566
PRA Class I and II Positive	1 (1.69%)	0	0.402
Total Positive PRA	5 (6.78%)	3 (7.31%)	0.713

There were 52 women and 48 men among the 100 individuals with AS diagnoses in the research. The mean age of the male and female patients was 34.74±13.36 years and 38.72 ± 14.17 years, respectively. Of the patients who were female, none found positive for PRA class I; four found positive for PRA class II, and four found positive for both PRA class I and II. One patient was positive for PRA class I, one for PRA class II, and two for both PRA class I and II in the case of the male patients. The statistical analysis revealed that the results of the PRA screening test did not significantly differ between males and females in AS. PRA screening results and a thorough statistical analysis are shown in Table 2.

Table 2: Number of AS patients, mean age, and results of PRA screening.

	Female (n=52)	Male (n=48)	p
Number of participants	52	48	-
Mean Age (Years)	38.72±14.17	34.74±13.36	-
Positive PRA Class I	0	1 (2.08%)	0.296
Positive PRA Class II	4 (7.69%)	1 (2.08%)	0.199
PRA Class I and II Positive	4 (7.69%)	3 (6.25%)	0.778
Total Positive PRA	8 (15.38%)	5 (10.41%)	0.278

Although statistical analysis did not reveal statistically significant differences, numerical differences were noted between the AS patients and the control group in the groups positive for PRA class II and both PRA class I and PRA class II. Details of the results are offered in Table 3.

Table 3: Comparison of the AS patient group's and the control group's PRA screening findings.

	AS Groups (n=100)	Control Groups (n=100)	p
Positive PRA Class I	1 (1%)	4 (4%)	1.00
Positive PRA Class II	5 (5%)	3 (3%)	1.00
PRA Class I and II Positive	7 (7%)	1 (1%)	0.783
Total Positive PRA	13 (13%)	8 (8%)	0.847

There were 49 male and 51 female patients with Behçet's disease overall in the study. Male patients had a mean age of 36.55±12.03 years, whereas female patients had a mean age of 34.37± 9.94 years. Just two female patients among those with Behçet's disease tested positive for PRA class II, and two patients tested positive for both PRA class I and PRA class II. No patient tested positive for PRA class I alone, according to the findings of the PRA screening. There was one positive PRA class I patient, five positive PRA class II patients, and one positive patient for both PRA classes I and II among the male patients. The statistical analysis revealed that there was no discernible difference in any group's PRA screening test results between males and females (Table 4).

Table 4: Number of patients of Behçet's disease, median age, and results of PRA screening.

	Female (n=49)	Male (n=51)	p
Number of participants	49	51	-
Mean Age (Years)	34.37±9.94	36.55±12.03	-
Positive PRA Class I	0	1 (1.96%)	1.00
Positive PRA Class II	2 (4.08%)	5 (9.8%0)	0.437
PRA Class I and II Positive	2 (4.0%8)	1 (1.96%)	0.614
Total Positive PRA	4 (8.16%)	7 (13.72%)	0.374

While there were numerical differences in the PRA class II and both the PRA class I and PRA class II groups when comparing the BD patient group to the control group, no statistically significant changes were found (Table 5).

Table 5: Comparison of PRA screening results between the control group and the BD group.

	BD Groups (n=100)	Control Groups (n=100)	p
Positive PRA Class I	1 (%1)	4 (%4)	1.00
Positive PRA Class II	7 (%7)	3 (%3)	0.197
PRA Class I and II Positive	3 (%3)	1 (%1)	0.851
Total Positive PRA	11 (%11)	8 (%8)	0.570

The research included 60 individuals with type 1 diabetes, among whom 33 were females and 27 were males. The average age of female patients was 30.7 ± 12.2 years, while that of males was 28.8 ± 11.5 years. Screening of PRA findings revealed that among female patients, one tested positive for PRA class I, one for PRA class II, and one for both PRA class I and II. In male patients, one tested positive for PRA class I, two for PRA class II, and one for both PRA class I and II. The results of the PRA screening test showed no significant variation among males and females across all categories according to statistical analysis (Table 6).

Table 6: PRA screening findings, mean age, and number of T1D patients.

	Female (n=33)	Male (n=27)	p
Number of participants	33	27	-
Mean Age (Years)	30.7±12.2	28.8±11.5	-
Positive PRA Class I	1 (3.03%)	1 (3.7%)	1.00
Positive PRA Class II	1 (3.0%3)	2 (7.4%)	0.860
PRA Class I and II Positive	1 (3.03%)	1 (3.7%)	1.00
Total Positive PRA	3 (9.09%)	4 (14.8%)	0.690

Table 7: Comparison between the T1D patient group and the control group's PRA screening findings.

	T1D Groups (n=100)	Control Groups (n=100)	p
Positive PRA Class I	2 (%3.33)	4 (%4)	1.00
Positive PRA Class II	3 (%5)	3 (%3)	1.00
PRA Class I and II Positive	2 (%3.33)	1(%1)	0.851
Total Positive PRA	7 (%11.66)	8 (%8)	1.00

There was no statistically significant difference seen between the three groups when compared to the control group (Table 7).

Every patient with an AS, BD, or T1D diagnosis had data gathered about them, such as when they were first diagnosed, the drugs they were taking to treat the condition, any pregnancy histories for female volunteer patients, any prior organ transplant history, and whether or not they had ever received blood transfusions. It was concluded, therefore, that the data gathered produced no variations that would have an impact on the PRA screening outcomes.

The mean PRA class II MFI values of all three patient groups were found to be greater than those of the control group when the MFI values of the patient groups were compared with the group under study. Moreover, the MFI values of the AS and BD groups were found to be greater than the control group, whilst the MFI values of the T1D patient group were found to be lower, when the PRA class II MFI values of the three patient groups were compared with those of the control group. Nevertheless, the statistical analysis revealed no discernible variation (Table 8).

Table 8: Comparison of the control group's PRA Positive MFI values with those of AS, BD, and T1D patients.

	PRA Class I MFI (min-max)	PRA Class I MFI Means	p	PRA Class II MFI (min-max)	PRA Class II MFI Means	p
Control	1148-1722 (n=4)	1468.67	-	1009-2163 (n=4)	1601	-
AS	1216-6818 (n=8)	3636.47	0.197	1204-9142 (n=12)	3223.99	0.521
BH	1081-5095 (n=4)	2774.52	0.718	1126-5948 (n=10)	2711.51	0.862
T1D	1159-1920 (n=4)	1585.75	1.00	1248-1717 (n=5)	1459.67	1.00

4. Discussion

The cause of AS, a persistent inflammatory illness, is not entirely known. In a 1973 study, the correlation between AS and HLA-B27 was initially discovered. A total of 407 HLA-B27 subtypes have been identified as a result of further studies that sought to investigate the link between AS and HLA. Of these, subtypes B27:03, B27:07, B27:06, B27:27, B27:29, and B27:47 were determined to be protective factors, while subtypes B27:04 and B27:15 to be risk factors. Further research revealed no correlation between AS susceptibility and subtypes B27:01, B27:02, B27:05, B27:08-15, B27:17-20, B27:23-24, B27:33, B27:35, B27:40, B27:46, B27:49, and B27:67 [10–14].

Behçet's disease is a systemic vasculitis that is located at the nexus of autoimmune and autoinflammatory disorders. It is typified by recurrent aphthous ulcers and ocular inflammation, and it can also occasionally affect the central nervous system, large vessels, joints, lungs, kidneys, and gastrointestinal tract [15, 16]. Mizuki as al. (2000) and Gül et al. (2001) have conducted studies on Behçet's disease and found that patients who test positive for HLA-B51 and HLA-B57 had higher rates and more severe cases of vaginal ulcers, cutaneous symptoms, positive pathergy tests, and ocular diseases. Moreover, research has shown that HLA-B35 functions as a protective factor. The phenotypic frequency of HLA-B51 has been reported to be increased by the MICA-A6 allele from non-HLA genes [17]. Additionally, mutations in IL-23 and IL-10 have been linked to an increased risk of developing Behçet's disease [18]. Furthermore, ERAP1 and HLA-B51 have been discovered to positively correlate in recent research [19].

T-cell-mediated death of pancreatic beta cells is the cause of type 1 diabetes (T1D), an autoimmune and complex hereditary illness [20]. According to Al-Terehi et al. (2016), there is also a belief that the disease develops as a result of the interaction between hereditary and environmental variables. Research examining the relationship between HLA and type 1 diabetes has revealed that certain genotypes and alleles, including HLA-DR09/DQ09, HLA-DR17/DQ02 [21], HLA-DQB103, and HLA-DQB102 [22], may be associated with a higher risk of type 1 diabetes, whereas other genotypes and alleles, including HLA-DR07/DQ09, DR15/DQ06 [21], HLA-DQB106, and HLA-DQB103/HLA-DQB1*06 [22], may offer protection against type 1 diabetes.

Looking over these three papers, it is clear that their primary focus is on the relationship between HLA and diseases; however, our research has not come across any studies that address the development of anti-HLA antibodies. Each of the three disease categories that were identified for our study was compared to the control group independently after PRA screening tests were administered to patients who met the internationally recognized diagnostic criteria. Compared to the control group, none of the disease groups showed any statistically significant differences in the comparison. Strong infections that occurred before blood collection, allergic reactions, or unidentified illnesses could all be causes of positive PRA screening test results in the control group.

The recipient's HLA antibodies are the largest barrier to organ transplantation. These antibodies are detected through the PRA screening test, which determines the suitability of the organ transplant candidate. The body's defensive mechanism creates antibodies against its own antigens in autoimmune disorders. If a person with an autoimmune condition is a candidate for an organ transplant, the creation of HLA autoantibodies may cause organ rejection. In order to avoid this, it's critical to find out if the patient has any autoimmune illnesses when gathering epicrisis data for organ transplantation. To avoid potential organ rejection, an autoimmune condition should be examined more thoroughly and its risks taken into account if it is present.

5. Conclusion

Autoimmune illnesses have been shown to produce autoantibodies in numerous investigations; however, it remains unclear if these autoantibodies are related to the human leukemia antigen (HLA). In this context, a prior study found positive in PRA class II screening tests in patients with RA after examining the literature. We created a study to ascertain the presence of PRA in three distinct autoimmune disorders in an effort to respond to the question, "Are there developments of anti-HLA antibodies in common autoimmune diseases?" We are the first to look into the relationship between AS, BH, and T1D illnesses and anti-HLA antibodies. Results were not statistically significant, despite a numerical difference between the control group and the illnesses. However, given the significant numerical disparities found, more care should be taken to find out if these illnesses are present while gathering a patient's medical history when they are about to have an organ transplant. A PRA screening test was employed as the analysis approach in this investigation. More precise PRA identification tests, such as LSA or SAB, which are more precise than the Luminex PRA screening test, can be employed to overcome these problems. We were unable to add LSA or SAB tests in our study due to financial restrictions. In the future, PRA identification techniques like LSA or SAB may be used to increase the sensitivity of the study. Furthermore, data from multiple centers' repeated research with higher sample sizes might be examined.

Limitations of the Study

Due to insufficient budget in our study, we were unable to include tests such as SAB and LSA. In future studies, it may be possible to plan more advanced research by incorporating highly sensitive PRA identification tests like SAB and LSA, and by increasing the sample size.

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Conflict of Interests

The authors declare no conflict of interest.

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Author Contributions

Conceptualization, H.D. and M.K.; methodology, H.D. and M.K.; data curation, M.K and H.D.; Formal analysis, M.K. and H.D.; funding acquisition, H.D. and M.K.; writing-review and editing, H.D. and M.K.

All authors have read and agreed to the published version of the manuscript.

Ethical Approval

All participants gave informed consent and the local institutional ethics committee approved the study methods (Date: 30.06.2022 no: B.30.2.ATA.0.01.00/549)" 'Declarations Section'-'Ethics approval and consent to participate' sub-section.

We declare that written consent has been obtained from all participants.

Data sharing statement

The data presented in this study are available on request from the corresponding author.

Consent to participate

Consent was obtained from the patients participating in the study.

Informed Statement

Informed consent was obtained from all subjects involved in the study.

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Transplantation Genetics: The Importance of non-HLA Antibodies, Genetic Insights and Future Perspectives

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Abstract: Solid organ transplantation is an effective and life-saving treatment method for patients with end-stage organ failure. Immunological evaluation is of great importance in the achievement of transplantation, and the determination of HLA incompatibility forms the basis of immunological transplantation success. The presence of donor HLA-related antibodies is known to be asssociated with graft loss and decreased survival rates. However, recent studies have shown clinical and experimental findings related to the important role of non-HLA antibodies in the antibody-mediated rejection and chronic rejection process. The mechanisms triggering auto-antibody production in organ transplantation are an important area of research. These mechanisms stimulate auto-antibody production in the patient causing the expression of organ-origin autoantigens such as ischaemia-reperfusion damage, surgical trauma, alloimmune responses, soluble antigens, extracellular vesicles and apoptotic bodies. In patients with a risk of non-HLA antibody-mediated rejection, there is an urgent need to determine the recipient immunological phenotypes, both for the development of targetted treatments and to increase both graft and patient survival. The aim of this review was to evaluate non-HLA antibody types and their effects on transplantation. ©2024 NTMS.

Keywords: non-HLA Antibodies; Solid Organ Transplantation; Transplantation Genetics.

1. Introduction

The detection of foreign structures in donor cells by the adaptive immune system of the patient constitutes the main immunological obstruction in organ transplantations. Human leukocyte antigens (HLA), which are located on the p arm of the 6th chromosome and encoded in the HLA complex, are accepted as the most important alloantigens in transplantation [1]. The determination of HLA incompatibility forms the basis of immunological transplantation success. The widespread clinical use of anti-HLA antibodies significantly increases transplantation success.

The presence of donor HLA-related antibodies is known to be associated with decreased survival rates and graft loss. It has been suggested that the transplantation outcome is affected synergistically by non-HLA antibodies together with the harmful effects of HLA antibodies. The damage caused to the transplantation process by HLA antibodies may cause the emergence of neo-antigens, and this can result in the production of antibodies against non-HLA antigens. However, the presence of non-HLA antibodies can increase the risk of the patient developing HLA-specific antibodies. All these findings emphasize the importance of determining the immunological risk of the patient by classifying non-HLA and HLA antibodies [2].

Despite improvements in immunosuppression regimens and the optimisation of patient management,

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antibody-mediated rejection remains a major impediment to long-term survival [3]. The identification of HLA-specific antibodies against donor HLA class I and II antigens has become a priority at this stage. With commercially developed tests and techniques in this field, there is a greater level of knowledge about the specificity, power, and function of these antibodies. From the correlation of antibody information obtained from clinical transplantation outcomes, it has been reported that patients with antibodies to donor HLA are faced with antibody-mediated rejection and worse transplantation outcomes [4]. Despite the histopathological findings of allograft dysfunction and antibody-mediated rejection during biopsy, antibodies specific to donor HLA have not been determined [4.5]. For example, in heart transplantations, non-detection of donor-specific HLA antibodies that cannot be detected in peripheral blood has prioritised the research of non-HLA antibodies, many of which are expressed by the vascular endothelium and often emerge after stress or transplant injury [5]. Although the determination of non-HLA antibodies is difficult, antibodies to non-HLA antigens have been shown to be related with transplant dysfunction or transplant rejection [6]. To date, the focus in clinical transplantation has been alloimmunity associated with HLA. However, the presence of antibody -mediated humoral rejection developing after kidney transplantation from siblings with compatible HLA shows the importance of non-HLA antigens of alloimmunity [7]. Terasaki et al. [8] reported that only 18% of kidney allograft losses may be associated with non-HLA independent immunological factors compared to 38% due to HLA mismatches [8]. Opelz et al. was reported the importance of the non-HLA alloimmunity response in transplantation success [9]. When the effect on the long-term results has been analyzed of the pre-transplant panel reactive antibody (PRA) levels of patients undergoing kidney transplantation from sibling donors fully compatible in HLA-A, HLA-B, and HLA-DR loci, the probability of incompatibility in other HLA loci has been reported (<3%, especially DQ and DP). From the findings obtained, the most noteworthy is that the PRA effect only becomes evident after the first year post-transplantation. Therefore, it has been concluded that non-HLA immunity has a stronger role than previously thought and non-HLA alloimmunity is usually associated with chronic allograft loss [9].

The development of antibodies against non-HLA autoantigens after organ transplantation is related with rejection and long-term survival. Although there is a lack of data about non-HLA antibodies, there are strong clinical and experimental findings that the antibodies determined play an important role in antibody-mediated acute and chronic rejection processes[9].

The mechanisms triggering autoantibody production in organ transplantation is an important area of research. Auto-antibody production in the patient is stimulated causing the expression of organ-origin autoantigens such as ischaemia-reperfusion damage, surgical trauma, alloimmune responses, soluble antigens, extracellular vesicles and apoptotic bodies. Th17 cells regulate autoantibody production, supporting the proliferation and maturation of autoreactive B cells in ectopic tertiary lymphoid tissue. Autoantibody-mediated graft damage can trigger alloimmunity and can cause the development of donor-specific HLA antibodies. In patients with a risk of non-HLA antibody-mediated rejection, there is an urgent need to determine the recipient immunological phenotypes, both for the development of targetted treatments and to increase both graft and patient survival [2].

The mechanisms of antibody formation

Non-HLA antibodies can be directed to auto or alloantigens, may be present before transplantation or may form *de novo* after tranplantation. There are thought to be various triggering events for autoantibody formation which can cause self-tolerance loss step by step. Inflammation can cause an increase in antigen expression and varying antigen processing and proteolysis [10-13]. In addition, post-translational modification, oxidative stress, and apoptosis may cause the formation of neoantigens [10]. Finally, the close relationship of infection agents and to their own peptides may cause cross-activation of autoreactive B and T cells [14]. By playing an important role in the selection of B cells directed against alloantigens, follicular helper T cells (Tfh) prevent the formation of autoreactive B cell clones. The irregularity of Tfh cells may cause humoral autoimmunity by causing impairment in B cell selection. Factors causing impairments in the eradication of immature autoreactive B cells through increased Th17 formation against allograft and/or donor HLA may further facilitate self-tolerance loss [15,16] (Figure 1).

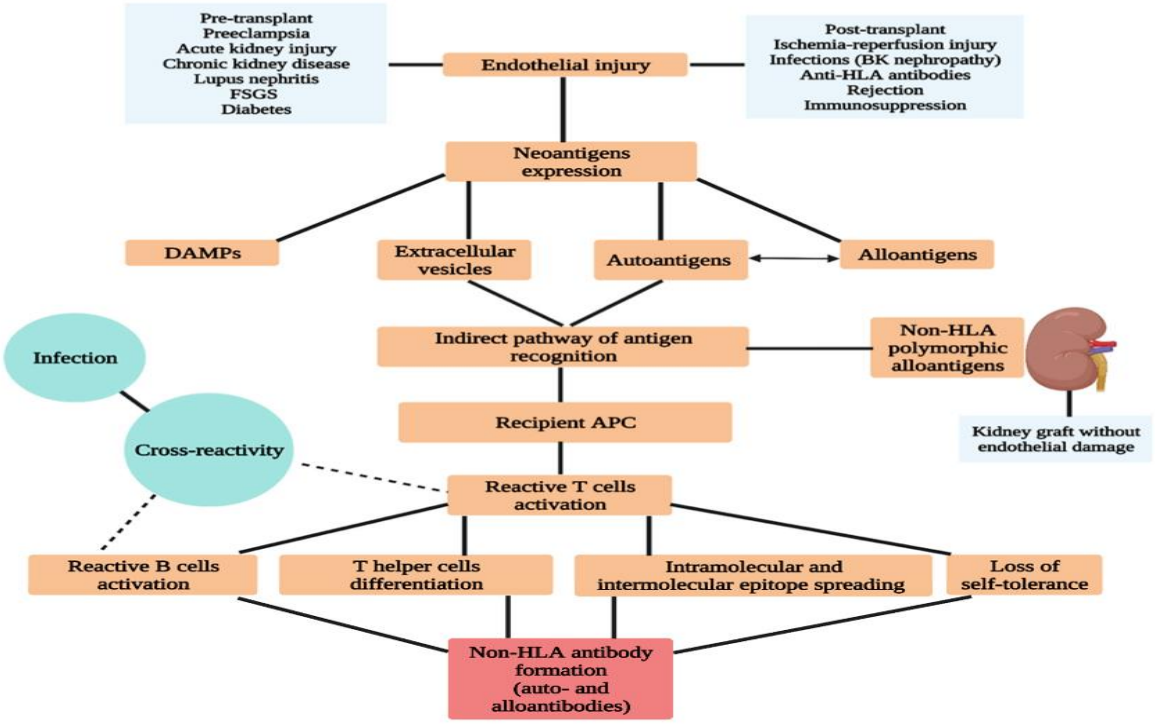

Figure 1: The mechanism of non-HLA antibody formation. FSGS: focal segmental glomerular sclerosis, BK: BK virus, HLA: human leukocyte antigen, DAMPs: Damaging molecular patterns, APC: antigen presenting cells [73].

The mechanism of non-HLA mediated graft damage

There is thought to be a synergistic effect of HLA and non-HLA antibodies. While HLA antibodies can reveal endothelial damage causing autoantibody formation and subsequent autoantigen exposure, the inflammatory response triggered by non-HLA antibodies can make the allograft more vulnerable to alloimmune response by increasing HLA expression [17]. This hypothesis is supported by several studies showing that patients with HLA and non-HLA antibodies have lower graft survival rates than patients with only one of these [18]. However, there are also studies in literature stating that in patients with both HLA and non-HLA antibodies, there is no relationship between these antibodies and graft survival [19]. Most autoantigens have been determined not only in the kidneys, but also in most other solid organs of the body [20]. To create a response, these antibodies are activated with highly specific mechanisms such as HLA antibody binding or ischaemia-reperfusion damage. Non-HLA antibodies do not directly cause major graft damage, because hyperacute rejection stimulated by these antibodies rarely occurs [21]. Several hypotheses have been suggested to explain non-HLA antibody-mediated graft damage, but the mechanisms have not been fully clarified (Figure 2).

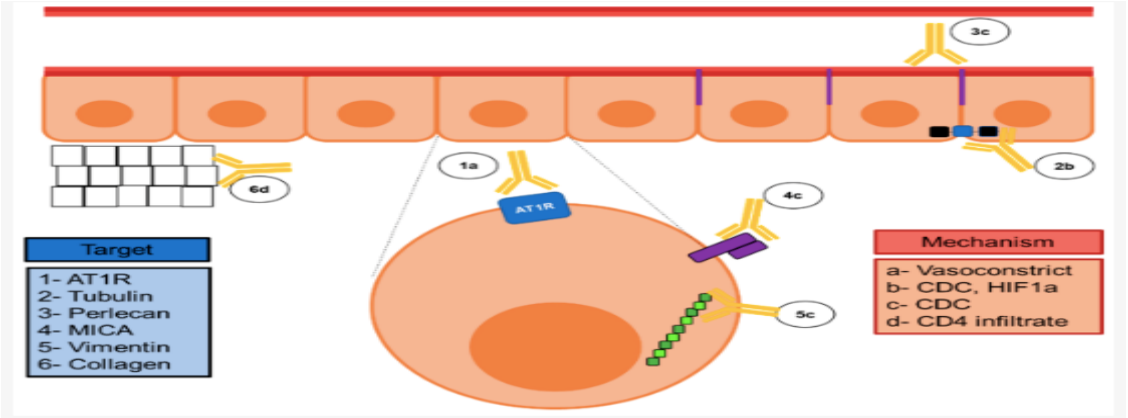

Figure 2: The mechanisms of non-HLA antibodies in solid organ transplantation. The structures containing blood vessels (red) and endothelial lining of cells (orange), extracellular matrix (collagen) and intercellular gap connections (black and blue) are shown. It is indicated how the antibodies are linked to intracellular components (eg.tubulin) and different membrane receptors with a single cell. The antibody targets are listed in the blue box, and the mechanisms associated with the target in the red box. The corresponding targets and mechanisms are defined in the white squares. Abbreviations: AT1R: Angiotensin 1 receptor, MICA: MHC class I-related chain gene A, CDC: Complement dependent cytotoxicity, HIF-1α = Hypoxia-inducible Factor 1 alpha [74].

Types of non-HLA Antibodies

Antibodies to MICA

Major histocompatibility complex (MHC) class I-related chain A (MICA) antigen is a polymorphic glycoprotein, encoded by the gene known as MHC class I chain within the HLA complex on the 6th chromosome. While the MICA molecule is expressed in monocyte, dendritic cells, endothelial and epithelial. It is shown that not found in T and B lymphocytes [22]. MHC class I chain-related gene A belongs to the HLA gene family. MICA protein has a structure similar to HLA class I, but is not associated with β2 microglobulin on the cell surface and shown that cannot bind to peptides [23]. Just like HLA molecules, they may carry different recipient and donor MICA alleles. The donor may develop antibodies against donor-specific MICA alleles [24]. The effect of MICA antibody on transplantation pathogenesis has been reported in kidney transplantations [19]. Patients with donor-specific MICA antibody are at higher risk of antibody-mediated rejection [5]. MICA is located in the interface between the allograft and the recipient blood and cannot be determined in silent endothelial cells directly targetted by the immune response. MICA can be stimulated by stress factors or cytokines such as TNF-α [25]. Previous studies have shown that MICA expression in tumour cells leads to the activation of NK cells mediated by the interaction of MICA/NKG2D and this causes cytotoxic protein and IFN-γ expression [23] (Figure 3).

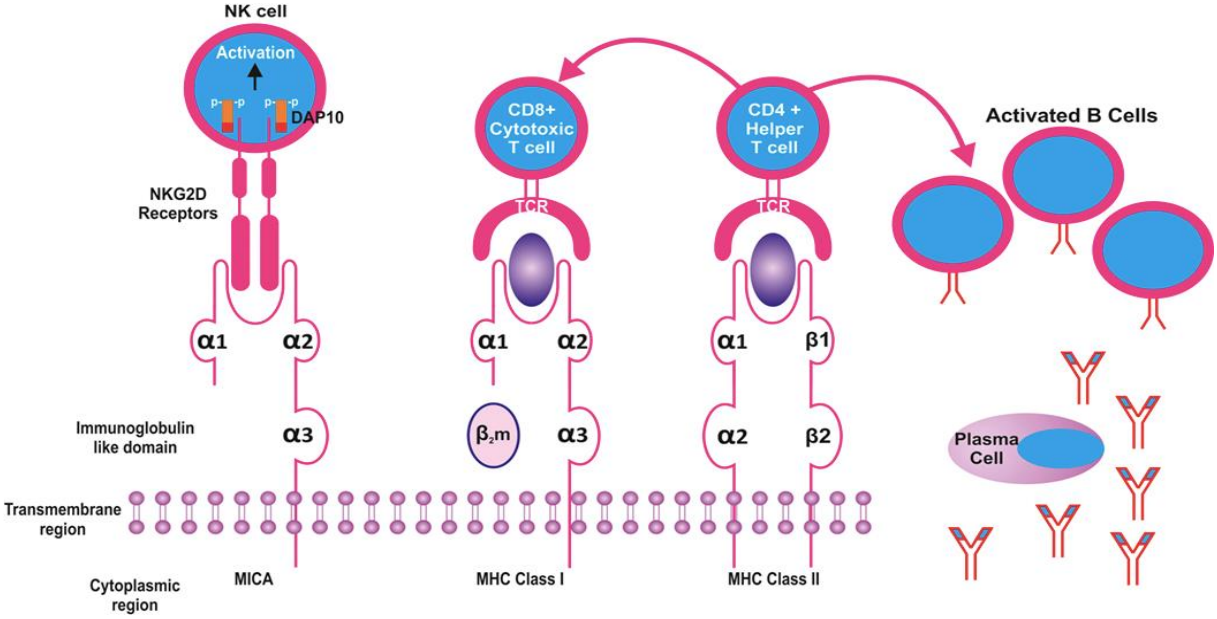

Figure 3: Stractural Similarities Between Major Histocompatibility Complex (MHC) Class I and II Molecules with MICA. The latter is equivalent to the heavy chain of MHC class I molecule without the β2 microglobulin. While the MHC I and II present peptides to CD8 and CD4 cells, respectively, the MICA recognizes NKG2D receptors on the surface of natural killer (NK) cells [22].

Antibodies against G Protein-Coupled Receptors (GPCRs)

AT1R (angiotensin II receptor type I) and ETAR (endothelin-1 type A receptor) belong to the GPCR family of the seven-transmembrane domain. As AT1R and ETAR are expressed on the cell surface and because of the extracellular regions where antibodies can be reached, the development of antibodies against these may be possible. Some antibodies such as AT1R have been reported to play a role in the pathophysiology of pre-eclampsia in pregnancy and autoimmune diseases such as systemic sclerosis [26-28]. There are several potential mechanisms to explain how these antibodies could develop in patients without any autoimmune disease. One of the possible causes may be immune suppression or the underlying inflammatory process itself causing the condition that cannot be tolerated. Another reason may be the separation of proteins such as Von Willebrand factor to smaller peptides, triggered by procedures such as dialysis [29]. Just as shear stress may cause AT1R to break from the cell surface and thus be exposed to neo-antigens, it may also disrupt the extracellular cycle of AT1R. The severity of damage of AT1R antibodies can be affected by the level of different AT1R isotypes expressed in the allograft. The effect in clinical findings of anti-AT1R antibodies has been defined in a group of kidney transplantation recipients with malignant hypertension [30]. This shows that AT1R antibodies such as AT1R binding with angiotensin II can promote vasoconstriction, H_2O intake, and sodium confinement, and can increase blood pressure [31]. Just like HLA antibodies, AT1R antibodies have a negative effect on the survival of transplanted organs. Although the presence of AT1R antibodies is associated with antibody-mediated rejection in kidney transplantations, they are not related with cellular-mediated rejection [32]. By creating a synergy with HLA antibodies, AT1R antibodies create a predisposition to transplantation rejection. The presence of AT1R antibodies which are

strongly bound and HLA class II donor-specific antibodies may be a reason for the acceleration of kidney transplantation rejection, hypertensive encephalopathy, and shorter survival following transplantation [32,33]. In addition, the harmful effect of AT1R antibodies on the graft does not require complement system activation. Reinsmoen et al. [32] reported that CD4 positivity was determined in the biopsy of only 1 of 6 patients with strong AT1R antibodies and the graft rejection was antibody mediated. In parallel with that study, Fuss et al. [34] reported that in cases with acute antibody-mediated rejection with CD4 negativity proven in biopsy according to Banff 2013, AT1R antibodies were determined in 11 cases, unlike donor-specific HLA antibodies. AT1R also plays an important role in glucose metabolism [35]. It has been suggested that increased expression of AT1R is associated with an increased risk of diabetic nephropathy and that AT1R blockage is effective in the treatment of diabetic nephropathy. However, there is a need for further studies in this field (Figure 4).

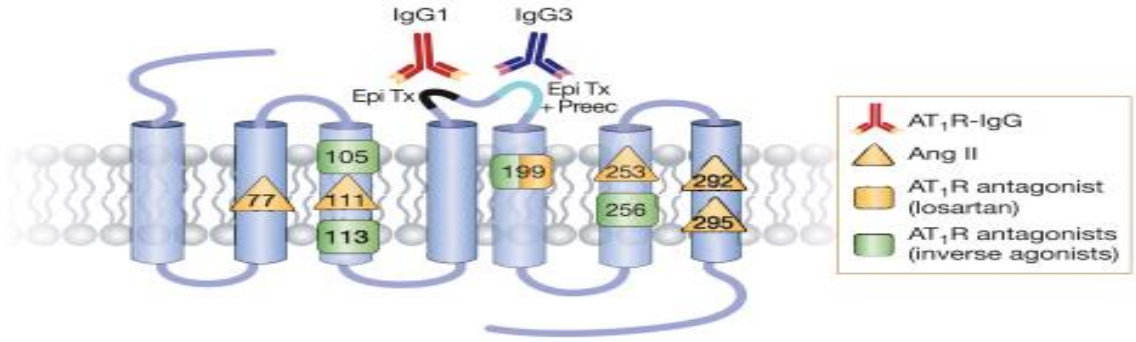

Figure 4: Key amino acids for the binding of receptor modulators are shown. The receptor is bound to Ang II, an orthosteric ligand, angiotensin II, transmembranes 2, 3, 6, and 7. AT1R antibodies as allosteric ligands are bound to the extracellular cycle 2 (ECL2) on 2 different epitopes (Epi) related to EpiTx and EpiTx+Preec acute graft rejection. Losartan is bound to the Angiotensin II orthosteric domain within transmembrane 5 (TM5). Other receptor antagonists are bound to receptors on transmembrane 3, 5, and 6 (TM3, 5, and 6). Preec: pre-eclampsia; Tx: transplantation [30].

Antibodies to Vimentin

Vimentin is a cytoskeletal type III intermediate filament protein, which is expressed in T cells, endothelial cells, neutrophils, fibroblasts, thrombocytes, smooth muscle cells, epithelial cells [36]. Vimentin is a molecule generally found within the cell. However, by being expressed on the cell surface in conditions such as endothelial damage or apoptosis, it becomes an immunogenic auto-antigen. This stimulates the formation of anti-vimentin auto-antibody [37]. Antibodies developing against vimentin play a role in the pathogenesis of these autoimmune diseases. Antibodies to vimentin have been associated with an increased risk of cardiovascular disease in some populations, but the mechanism of this relationship has not been fully clarified.

Vimentin, which is a sub-unit of the intermediate filament is important for the stabilisation of cytoplasm as a cytoskeletal component. It has been reported that it is expressed by macrophages, endothelial cells, vascular smooth muscle cells, active thrombocytes, apoptotic T cells and neutrophils [38]. While expression is increased with proinflammatory cytokine TNF-α, it is inhibited by anti-inflammatory cytokine IL-10. This suggests that vimentin could be important in immune response [39]. Vimentin antibodies have been determined in the pre-transplant serum of patients with kidney failure [40]. In a previous study, it was reported that IgM increased antibody titers against vimentin every year compared to pre-transplant titers. However, no difference was found between patients with interstitial fibrous and tubular atrophy and the kidney recipient control group, although IgG vimentin antibody was determined at a high level in the patients with interstitial fibrous and tubular atrophy. These results suggest that IgG antibodies in patients with interstitial fibrous and tubular atrophy could be related to the diseases pathogenesis, but there is no effect of IgM antibodies [41].

Anti-Endothelin A Receptor Antibodies

Endothelin A receptor (ETAR) is a transmembrane G-protein-related receptor, which plays a role in providing the balance of blood pressure and sodium and is encoded by the ENDRA gene on the 4th chromosome [42]. ETAR can be found in immune cells and is expressed primarily in vascular endothelial cells, mesengial cells, tubular epithelial cells and vascular smooth muscle cells [43]. Anti-ETAR antibodies are IgG1 subtype autoantibodies and show a similarity to the mechanism of formation of anti-AT1R antibodies [44]. Previous studies have reported that anti-ETAR antibodies are associated with ABMR, vascular rejection, and deteriorating graft function and graft loss following kidney transplantation [45,46].

Anti-ARHGDIB (Rho Guanine Nucleotide Exchange Factor 2) Antibodies

ARHGDIB is an intracellular GTP-binding protein, which is expressed in different tissues and organs and

plays a role in several cellular activities. Expression in kidney graft is affected in normal or pathological conditions. In a kidney graft when abnormality is not determined histologically, ARHGDIB expression is seen less in the endothelial cells in interlobular arteries, peritubular capillaries and glomerular capillaries. In contrast, in a kidney graft with acute tubular necrosis, ARHGDIB expression has been determined to be greater in the endothelial cells in interlobular arteries, peritubular capillaries and glomerular capillaries [47]. In a large-scale cohort study, antibodies to ARHGDIB were determined to have a significant effect on graft loss independently of anti-HLA DSA [47]. Endothelial damage triggered by ischaemia-reperfusion damage has been reported to trigger ARHGDIB expression and autoantibody formation [47]. ARHGDIB is considered to be a minor histocompatibility antigen, we examined the relation between antibody levels and potentially immunizing events, but found no link with repeat transplantation, female sex, pregnancies, or potentially confounding factors such as diabetes type 1, or several primary renal diseases [47]. In another study, anti-ARHGDIB antibodies determined in anti-HLA DSA positive patients were reported to be associated with an increase in graft damage [48]. However, there are also other studies in literature stating the opposite. Betjes et al. reported that increased anti-ARGHDIB auto-antibody expression in patients with chronic ABMR was not related to graft survival [49].

Anti-PECR (Peroxisomal Trans-2-Enoyl-CoA Reductase) Antibodies

PECR is a trans-2-enoil-CoA reductase specific to peroxisomal NADPH, which catalyses the reduction of trans-2-enoil-CoA with chain length varying between 6:1 and 16:1 [50]. PECR expression increases due to graft damage in kidney transplantation. It has been reported to be associated with the development of glomerulopathy and biopsy-proven ABMR [51].

Anti-PRKCZ (Protein Kinase C Zeta Type) Antibodies

PRKCZ is a type of protein kinase C, associated with proliferation, apoptosis, survival, and inflammation. Over-expression of PRKCZ has been reported in an ischaemia-reperfusion study [52]. In a study of paediatric renal transplant patients, although it was thought that graft rejection was related to antibodies to PRKCZ, it was concluded that there is insufficent data on this subject yet [53].

Anti-Agrin Antibodies

Agrin is a heparan sulfate proteoglycan, which is expressed at a high level in glomerular basal membranes. The C-terminal fragment (CAF), which is a fragmentation product of agrin, has been utilized as a potential biomarker for graft function in patients with renal transplantation. CAF has been reported to be related to creatinin, cystatin C, estimated glomerular filtration rate, and both early period and delayed graft function following kidney transplantation [54,55]. In another study, CAF was utilized as a risk factor for proteinuria and kidney graft loss in patients with transplant glomerulopathy [56].

Anti-Myosin Antibodies

Myosins are a series protein family which bind to the actin cytoskeleton and transport protein through ATP hydrolysis. The human and rat thymus does not express myosin heavy chain proteins and therefore, CD4+ T cells cannot be selected as negative for myosin in the maturation process [57]. As a result of this mechanism, it may be associated with autoimmune myocarditis, which is frequently associated with autoantibodies [57]. The presence of myosin antibodies has been associated to antibody-mediated rejection in heart transplants and the development of chronic allograft vasculopathy [58]. Although many single nucleotide polymorphisms of cardiac myosin have been identified, it is still not clearly known whether the myosin antibodies determined in patients are donor-specific or not.

Anti-Perlecan/LG-3 Antibodies

Perlecan is a heparan sulfate proteoglycan, which is found in vascular and epithelial basal membranes. Perlecan is an extracellular matrix proteoglycan, and an important component of the endothelial basal membrane, functioning as a barrier between the blood in circulation and the surrounding tissues. The CAF of perlecan contains LG3 regions (lamina-like globular areas) with high immunogenic properties [59]. In a rat transplant model, it has been shown to function as a co-receptor for fibroblast growth factor 2 to be able to stimulate cell proliferation [60]. Studies have also shown that vascular damage caused the release of apoptotic vesicle-like vesicles, and these were trriggered by the production of LG3 antibodies, which are the CAF of perlecan [59]. In kidney transplant patient, a high level of LG3 antibodies in both the pre- and post-transplant periods has been reported to be associated with acute vascular rejection [18]. Anti-LG3 antibodies are one of the factors increasing ischaemia-reperfusion damage, and it has been reported that these antibodies could be related with immune-mediated vascular rejection and delayed graft function within 1 year of transplantation [61]. Previous studies have also shown that memory B cells against LG3 are independent of T cells for the production of anti-LG3 antibodies, but the help of T cells is necessary for the production of anti-LG3 antibodies. T lymphocytes play an important role in anti-LG3 antibody production. This theory is supported by the decreased antibody level at the end of immunosuppressive treatment applied with calcineurin inhibitors [62].

Collagen

Late- term kidney allograft loss associated with chronic allograft nephropathy is one of the main problems threatening the long-term success of kidney transplantation. Transplant glomerulonephropathy is characterised by glomerular basal membrane

duplication and has been associated with chronic renal allograft rejection. The development of antibodies against collagen IV and fibronectin has been reported in kidney transplantation patients diagnosed with transplant glomerulonephropathy [63]. IFN-γ and IL-17 are expressed in these patients, and an increase has been determined in CD4+T cells specific to collagen IV and fibronectin, and a decrease in the cytokine IL-10 level. This indicates the role of collagen IV in the pathogenesis of chronic rejection [63].

HY Antigens

Proteins encoded in the Y chromosome have a sequence of similarity at the rate of 90% to homologs in the X chromosome. For example, RSP4Y protein (40S ribosomal protein S4, Y isoform 1) in the Y chromosome shows a difference with a change in 19 amino acids when compared to RSP4X variant which is based on a single nucleotide polymorphism [64]. Following the identification of HY-specific alloimmune T cells in transplants with gender mismatch, HY alloantibodies have also been determined to have been observed in individuals with acute allograft damage [65]. In a retrospective cohort study, long-term negative effects were reported of kidney transplantations made between different genders [66].

Other non-HLA Antibodies

The endothelium covers the interface between the graft and the recipient tissue. The first target of the antigens expressed by these cells is the recipient immune system. Antibodies against 4 non-HLA endothelial antigens have been determined in the serum of renal transplantation patients; endoglin, FMS-like tyrosine kinase-3 ligand (FLT-3) EDIL3, and ICAM4. Endoglin is a membrane glycoprotein expressed primarily in the vascular endothelium, which regulates angiogenesis and revascularisation [67]. FLT-3 is a tyrosine kinase that regulates cell differentiation, survival, and proliferation [68]. FLT-3 signal activation is thought to promote multiple myeloma angiogenesis [69]. EDIL3 is expressed by endothelial cells and is associated with the extracellular matrix. EDIL3 expression inhibits leukocyte-endothelial adhesion [70]. ICAM4 is known as a Landsteiner-Wiener blood group antigen. It is a one-way transmembrane protein and provides leukocyte binding by entering into interaction with integrin [71]. The presence of these antibodies has been associated with post-transplant donor-specific HLA antibodies, antibody-mediated glomerulopathy, and early transplant glomerulopathy [72] (Figure 5).

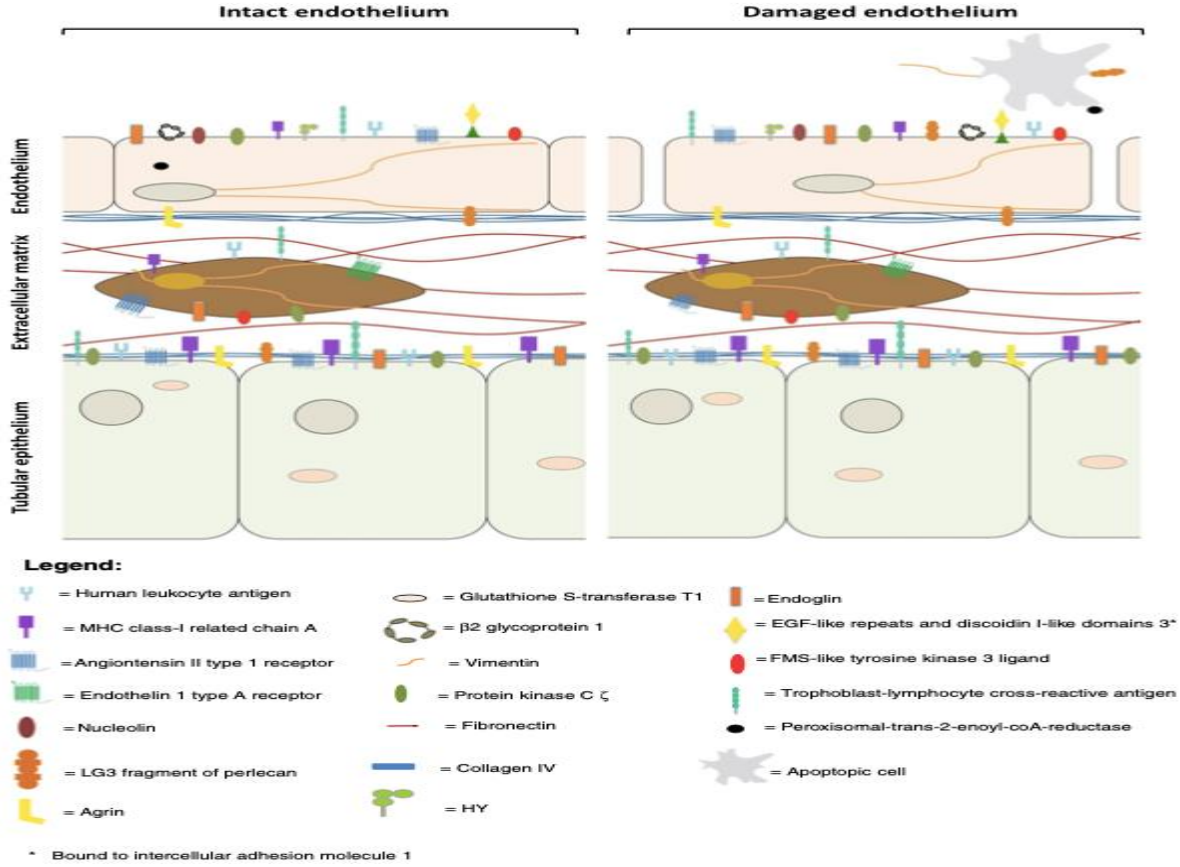

Figure 5: Localization of non-HLA antigen targets in the peritubular capillary in the quiescent state following endothelial damage [66].

Suggestions and Future Perspectives

Antibodies play a crucial role in transplantation, influencing both the success and potential complications of the procedure. The personalized characterization of antibodies is crucial in various fields such as biotechnology, biomedical research, and drug development. The fundamental steps of the characterization process include the binding properties to specific antigens, the impact of immunoglobulin structure on biological activity, functional activities such as cellular immune stimulation, post-administration immunogenicity and immune response, stabilization, post-translational modifications, and cross-reactivity. This process plays a critical role in the development of therapeutic antibodies and provides essential evaluation in clinical applications.

The evaluation of immunological risk in kidney transplantation is based on the evaluation of alloimmunity and anti-HLA antibodies. ABMR due to anti-HLA antibodies is evaluated with several different clinical tests. However, recent studies in this field have shown that immunological risk evaluation only focussed on anti-HLA donor-specific antibodies is insufficient. Moreover, in addition to the evaluation of non-HLA antibodies in respect of the immunological risk, it can also be of benefit in terms of graft survival, preventing graft rejection, and case management. There is increasing importance of non-HLA antibodies in addition to the classic HLA antibodies in renal transplantation. Many non-HLA antibodies have been identified in kidney transplantation patients, and with proteomic approaches, more potential antigen targets have become detectable. However, the full clinical importance of non-HLA antibodies in renal transplantation is prevented because of extremely heterogenic study designs with differences in test methods, immunsuppresive regimens, and result measurements. When the great differences in antibody incidence reported even in the same tests and the technical difficulties of the existing non-HLA antibody tests are taken into consideration, it can be seen to be important that efforts continue to be made on the subject of developing reliable and sensitive diagnostic tests. Moreover, the establishment of an antibody panel instead of a single antibody will be able to provide information about the role of non-HLA antibodies as an aid to defining the rejection and graft survival risk profiles. Non-HLA antibodies can be co-produced in certain types of transplants. Further studies in this area should be increased. The development and widespread use in clinical practice of a new Luminex-based test which will test more than one non-HLA antibody at the same time, will be extremely important in the success of graft survival.

2. Conclusions

Antibodies play a crucial role in transplantation, influencing both the success and potential complications of the procedure. The personalized characterization of antibodies is crucial in various fields such as biotechnology, biomedical research, and drug development. The fundamental steps of the characterization process include the binding properties to specific antigens, the impact of immunoglobulin structure on biological activity, functional activities such as cellular immune stimulation, postadministration immunogenicity and immune response, stabilization, post-translational modifications, and cross-reactivity. This process plays a critical role in the development of therapeutic antibodies and provides essential evaluation in clinical applications.

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Limitations of the Study

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MA: Conceived and designed the study, collected data and wrote the manuscript. EB, NA: Contributed to study design, supervised the work. All authors have read and approved the final version of the manuscript.

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None.

Data sharing statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent to participate
None.

Informed Statement
None.

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