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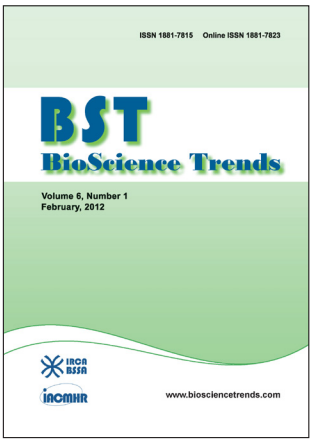

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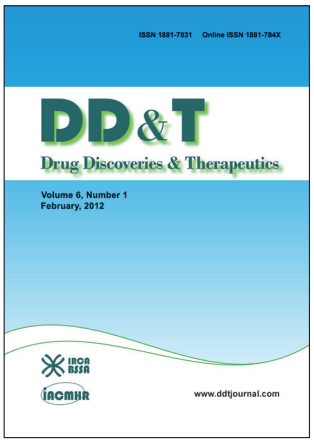

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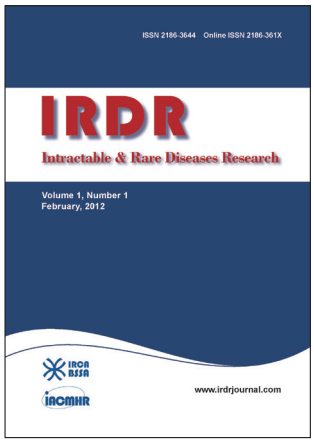

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A review of 99mTc-sestamibi SPECT/CT for renal oncocytomas: A modified diagnostic algorithm

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SUMMARY 99mTc-sestamibi SPECT/CT is a promising nuclear medicine imaging investigation for benign renal lesions such as renal oncocytomas. The purpose of this article is to *i*) review the current literature on 99mTc-sestamibi SPECT/CT, *ii*) to review to current application of 99mTc-sestamibi SPECT/CT for indeterminate renal lesion imaging, and *iii*) to discuss present limitations and areas for future research. The literature has been reviewed up to April 2022 for articles relating to the application of 99mTc-sestamibi SPECT/CT for benign renal lesions including a recently published systematic review and meta-analysis performed by the authors. One study evaluating 99mTc-sestamibi SPECT alone and five studies evaluating 99mTc-sestamibi SPECT/CT have been performed to date. 99mTc-sestamibi SPECT/CT demonstrates high sensitivity and specificity for detecting benign renal lesions, particularly renal oncocytomas. 99mTc-sestamibi SPECT/CT demonstrates near-perfect specificity for benign and low-grade renal lesions. The optimal quantified threshold ratio for tumor-to-background renal parenchyma radiotracer uptake for a positive result is > 0.6. In this article, we propose a modified diagnostic algorithm for small enhancing renal masses measuring 1-4 cm in which suspected benign lesions after conventional imaging are considered for 99mTc-sestamibi SPECT-CT. In this algorithm, positive studies can be monitored with active surveillance rather than requiring invasive biopsy and/or targeted therapy.

Keywords oncocytoma, RCC, renal, SPECT, sestamibi, surveillance

1. Introduction

The incidence of renal lesions has shown rapid growth in recent previous decades with an increasing number of surgical resections performed but no corresponding reduction in mortality (*1*). The rising incidence of incidental benign renal lesions on imaging is believed to play a considerable role in this result (*2,3*). Renal oncocytomas (RO) represent a particularly challenging benign renal lesion to differentiate from renal cell carcinomas (RCC) on imaging and frequently require surgical resection. In one study of nearly 3,000 surgically resected renal tumors, ROs accounted for 73% (274/376) of the surgically resected benign renal lesions (*4*).

One interesting histopathological feature previously found in ROs is a robust presence of mitochondria. In electron microscopy studies, the frequent presence of mitochondria in ROs has been a particularly distinguishable feature compared to chromophobe renal cell carcinomas (ChrRCC), a lesion which is essentially indistinguishable from RO on conventional imaging (*5,6*). This histopathological difference is believed to be exploited in 99mTc-sestamibi imaging, as sestamibi is a lipophilic cation which has been shown to accumulate in cells with high density mitochondria (*7*). Gormley *et al*. were the first group to hypothesize the potential application of 99mTc-sestamibi imaging with a pilot SPECT imaging study in 1996 (*8*). In their study, they performed 99mTc-sestamibi SPECT imaging on 6 patients including one oncocytoma, one renal cyst, one angiomyolipoma one cystic RCC and two solid RCCs. Of these patients, only the renal oncocytoma was shown to have a relative tumor-to-background renal parenchyma uptake of > 1 with a ratio of 1.44, nearly 0.6 greater than the next highest lesion.

Further exploration of 99mTc-sestamibi imaging remained relatively quiescent until 2015 when Rowe *et al*. published the first pilot study evaluating 99mTc-sestamibi SPECT/CT with 6 patients (3 renal oncocytomas and 3 RCCs), which showed complete differentiation of the relative tumor uptake between the groups (ROs 0.85-1.78 versus RCCs 0.21-0.26) (*9*). Since then, four additional studies have been

published in title or conference abstract format (*10-13*). One additional study has been posted on the Cochrane Central Register of Controlled Trials but remains unpublished to date (*14*). A systematic review and meta-analysis evaluating the diagnostic accuracy of 99mTc-sestamibi SPECT/CT in benign renal lesions such as renal oncocytomas has been recently published (*15*). The purpose of this article is to *i*) review the current literature on 99mTc-sestamibi SPECT/CT, *ii*) to review to current application of 99mTc-sestamibi SPECT/CT for indeterminate renal lesion imaging, and *iii*) to discuss present limitations and areas for future research.

2. Diagnostic performance for benign renal lesions

Five studies evaluating the diagnostic performance of 99mTc SPECT/CT for benign renal lesions with a total of 148 lesions are summarized in Table 1. A total of 31 ROs were included in reviews to date with 29 (94%) demonstrating positive sestamibi uptake. An additional 6 lesions were hybrid oncocytic/chromophobe tumors (HOCT), all of which were positive for sestamibi uptake. Only 3 of 8 (38%) ChrRCCs were positive for uptake and 2/98 (2%) of all other renal cell carcinomas (RCC) were positive for sestamibi uptake. A recent meta-analysis demonstrated a sensitivity and specificity of 92% (95% CI: 72-98%) and 88% (95% CI: 79-94%) respectively for RO versus other renal lesions and 86% (95% CI: 66-95%) and 90% (95% CI: 80-95%) for benign versus malignant lesions when HOCTs were considered malignant (*15*). The positive and negative likelihood ratios for benign versus malignant lesions were 8.6 (95% CI: 4.1-17.9) and 0.16 (95% CI: 0.06-0.42) respectively.

There is no clear consensus regarding the characterization of HOCTs. The 2013 Vancouver Classification of Renal Neoplasia by the International Society of Urological Pathology (ISUP) characterized HOCTs as a subcategory of ChrRCCs given the presence of some morphologic characteristics of ChrRCCs (*16*). However, the group also recognized that this characterization is not clear as in some cases such as patients with oncocytomatosis, HOCTs may in fact represent a morphologically distinct category and not a progressive spectrum between ROs and ChrRCCs. In 2016, the World Health Organization (WHO) significantly revised their fourth edition of the WHO "blue book" classification of urinary system and male genital organ tumors (*17*). In this most recent version, no specific classification of HOCTs as malignant is made. Gorin *et al*.'s group have chosen to consider HOCTs as benign renal lesions, citing a study following four HOCTs for 44 months without progression (*10,18*). Their group has recently performed a meta-analysis of 167 patients including unpublished data from their institution in which they characterize ROs and HOCTs together as benign lesions with a sensitivity and specificity of 86.6% (95% CI: 77.3-93.8%) and 89.1% (95% CI: 82.6-94.2%) respectively for 99mTc-sestamibi SPECT/CT (19). When HOCTs were characterized as benign lesions in a published meta-analysis, the sensitivity, specificity, and positive predictive value for benign renal lesions became 88% (29/33), 95% (80/84), and 88% (29/33) respectively (*15*).

Despite electron microscopy studies demonstrating a distinct difference in number of mitochondria between oncocytomas and ChrRCCs, 99mTc-sestamibi SPECT/CT is currently not specific at sub-classifying the two lesion types. This evaluation is limited however, by a small available sample size to date. ChrRCCs are also known to represent a more indolent form of RCC with better long-term prognosis than other RCC subtypes (*20*). Some authors have even argued that biopsy proven ChrRCC < 2 cm and deep (> 5 mm depth) 2-4 cm ChrRCCs should be managed with active surveillance rather than surgery (*21*). Of the 5 sestamibi positive malignant renal lesions noted to date, at least 3 are ChRCC with a third not subtyped and the fourth representing a papillary RCC. No clear cell RCCs have demonstrated sestamibi update on published articles to date.

3. Threshold value for a positive result

Three of five published studies have used semi-quantitative analysis demonstrating an optimal cut-

Table 1. Summary of patients in diagnostic performance studies evaluating 99mTc-sestamibi SPECT/CT for benign renal lesions

Authors (*Ref.*)	No. RO (No. positive)	No. AML (No. positive)	No. HOCT (No. positive)	No. ChrRCC (No. positive)	Other RCC (No. positive)	No. MA (No. Positive)	Lymphoma (No. Positive)
Gorin (*10*)	6 (5)	1 (0)	2 (2)	4 (2)	37 (0)		
Rowe (*9*)	3 (3)				3 (0)		
Sistani (*11*)	7 (7)		1 (1)	2 (1)	21 (0)		
Tzortzakakis (*12*)	12 (11)	1 (1)	3 (3)	2 (0)	11 (1)	1 (0)	1 (0)
Zhu (*13*)	3 (3)	1 (1)		Unclear*	26 (1)		
Total	94% (29/31)	67% (2/3)	100% (6/6)	38% (3/8)	2% (2/98)	0% (0/1)	0% (0/1)

RO: renal oncocytoma; AML: angiomyolipoma; HOCT: hybrid oncocytic/chromophobe tumors; ChrRCC: Chromophobe RCC; RCC: renal cell carcinoma; MA: metanephric adenoma. *, Conference abstract with no details of renal cell carcinoma subtypes provided.

off tumor-to-background renal parenchyma ratio of 0.6 (*9,10,13*). Tzortzakakis *et al*. utilized a visual analysis resulting in a sensitivity of 88% (15/17) and a specificity of 93% (13/14) for benign versus malignant renal lesions when HOCTs were considered benign (*12*). However, their group did recommend a more quantitative method of analysis to improve evaluation. A secondary analysis of Gorin *et al*.'s patients showed that quantitative analysis demonstrates a slightly increased but potentially clinically important differentiation between benign and malignant lesions, especially with lesions demonstrating an uptake ratio near the 0.6 cut-off (*22*). When quantitative methods are utilized, studies have shown excellent to near-perfect intra-observer and inter-observer agreement for diagnosis of a positive result with 99mTc-sestamibi SPECT/CT (*10,23*). Tzortzakikis *et al*. recently demonstrated that the intra-class correlation coefficient for SUVmax measurements by the same reader was 97-99% and 87-89% between readers for solid renal tumors. Strong agreement is likely at least partially related to large differences between tumor-to-renal parenchyma ratios between negative and positive results. Only a small proportion of the renal lesions evaluated to date have demonstrated ratios between 0.6-0.8.

4. Current application for indeterminate renal lesions

The literature currently demonstrates that 99mTc-sestamibi SPECT/CT is both sensitive and specific for identifying benign renal lesions at a cut-off tumor-to-renal parenchyma ratio of 0.6. The test becomes very specific when HOCTs are also characterized as benign renal lesions. Although the test is not good at differentiating ROs from ChrRCCs, very few non-ChrRCCs have demonstrated positive uptake on this examination, supporting a very high specificity for benign and low-grade renal lesions.

99mTc-sestamibi SPECT/CT is currently applicable for indeterminate renal lesions which are considered for active surveillance rather than surgical resection. In one retrospective study evaluating the added value of 99mTc-sestamibi SPECT/CT to conventional cross-sectional imaging, preoperative sestamibi imaging was shown to improve the confidence of a conventional imaging diagnosis in nearly 30% of cases (14/48) (*24*). In their study, the area under the curve increased from 0.60 for conventional imaging alone to 0.85 after combining conventional imaging with 99mTc-sestamibi SPECT/CT. Another review has supported this argument, suggesting that applying 99mTc-sestamibi SPECT/CT in indeterminate renal lesions < 4 cm will increase the number of patients undergoing active surveillance rather than unnecessary intervention (*25*). Therefore, we propose an imaging pathway in which small enhancing renal masses measuring 1-4 cm suspected to represent benign lesions after conventional imaging be considered for 99mTc-sestamibi SPECT/CT. In this pathway, lesions which demonstrate positive uptake have a high specificity for benignity and can be monitored with active surveillance rather than biopsy and/or targeted therapy. The proposed imaging pathway is demonstrated in Figure 1.

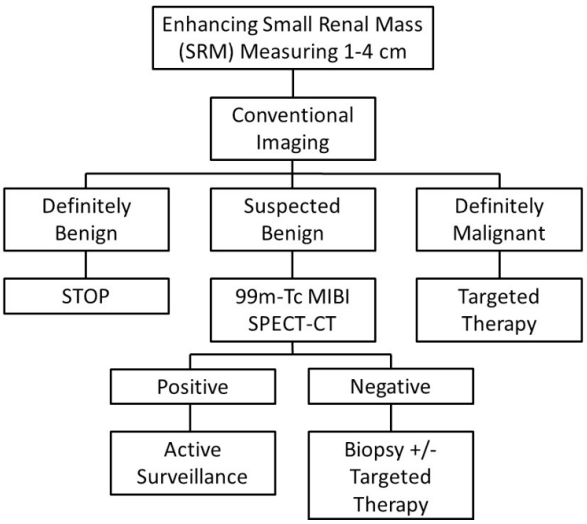

Figure 1. Proposed imaging pathway with integration of 99mTc sestamibi for small enhancing renal masses measuring 1-4 cm being considered for active surveillance.

5. Limitations and future research

The main limitation to date is a small sample size with only 148 lesions described in the literature. Given that the acquisition technique was similar amongst studies and the diagnostic criteria for most studies was also similar, variability amongst studies is lower than is typically seen in diagnostic accuracy studies. Specific imaging acquisition techniques for each study are demonstrated in Table 2. More studies from different institutions will help improve confidence in the diagnostic performance of this examination, particularly for patients eligible for active surveillance rather than intervention with our proposed pathway.

In addition to studies evaluating performance alone, subgroup analysis will aid in better characterizing which situations this study is best applied. For example, smaller lesions (< 1.5 cm) are generally known to have lower sensitivity on SPECT imaging due to limits in spatial resolution and partial volume averaging (*26*). Understanding differences in performance dependent on size will assist in knowing the minimum size criteria for 99mTc-sestamibi SPECT/CT application. Only two studies describe the mean lesion size, identifying an average lesion of 3.1 cm, although these are not subcategorized by positive and negative results. Tzortzakikis *et al*. report the number of tumors by size range noting that 19% (6/31) of their lesions were

Table 2. SPECT/CT acquisition details for individual studies

Authors (*Ref.*)	SPECT/CT Brand	Dose MIBI (MBq)	SPECT/CT Post Injection Timing (min)	Collimator Energy	Matrix Size	Projection Timing (sec)	Range of Projection	CT kV	CT mAs	CT Slice Thickness (mm)
Gorin (*10*)	Siemens*	925	75	NR	NR	NR	NR	NR	NR	NR
Rowe (*9*)	Siemens*	925	75	Low	64 × 64	28	180 degrees @ 3o Intervals	130	90	3
Sistani (*11*)	GE**	1110	60 - 90	NR	NR	NR	NR	NR	NR	NR
Tzortzakakis (*12*)	Siemens*	925 ± 25	60 - 90	Low	128 × 128	NR	NR	130	Modulated	5
Zhu (*13*)	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR

*Siemens Symbia, 16 Slice SPECT/CT; **GE Discovery, 16 Slice SPECT/CT; NR: Not Reported.

between 1-1.5 cm but do not clearly define which of these were positive or not. They did not include any lesions < 1 cm.

Another area for subgroup analysis would be specifically evaluating the diagnostic performance for other non-oncocytoma benign renal masses. For example, prior studies analyzing the ultrastructure of AMLs have identified numerous mitochondria in these lesions as well (*27*). In studies reported to date, 2/3 angiomyolipomas have demonstrated positive uptake. Lipid poor AMLs are another difficult lesion to diagnose with conventional imaging, and if this test can sub-classify these lesions, there may be additional value in using this examination in specific circumstances such as T2 hypointense indeterminate renal lesions (*28*).

Finally, an area currently being explored by the Johns Hopkins group is the utilization of 99mTc-sestamibi SPECT/CT in dual-tracer SPECT imaging (*29*). Several agents targeting the transmembrane protein carbonic anhydrase IX (CAIX) have been developed, including 124I-girentuximab, which has been trialed in a large multicenter study of 195 patients with PECT/CT (REDECT Trial) demonstrating a sensitivity of 86.2% (95% CI: 75.3-97.1) and specificity of 85.9% (95% CI: 69.4-99.9) for clear cell RCC, statistically better than the comparator contrast-enhanced CT (p = 0.005) (*30*). This combined tracer would have the potential to differentiate indeterminate renal lesions into benign renal lesions such as oncocytoma, but also further characterize 99mTc-sestamibi SPECT/CT negative lesions into clear cell RCC or other. The Johns Hopkins group has developed an 111In-labeled SPECT radiotracer-targeting CAIX and are currently investigating a dual-tracer SPECT study with 99mTc-sestamibi in a single center prospective trial (*29,31*).

6. Conclusion

Current literature has shown that 99mTc-sestamibi SPECT/CT is both sensitive and specific for benign and low-grade renal lesions such as oncocytomas and hybrid oncocytic/chromophobe tumors. We propose a diagnostic algorithm with the use of 99mTc-sestamibi SPECT/CT for small enhancing renal masses measuring 1-4 cm suspected to be benign, where studies with positive uptake can be monitored with active surveillance rather than undergo an invasive diagnostic and/or therapeutic procedure. Future studies evaluating the diagnostic performance of 99mTc-sestamibi SPECT/CT for indeterminate renal lesions with subgroup analysis and dual-tracer studies will continue to refine specific applications in indeterminate renal lesions.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Review

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Association of human gut microbiota with rare diseases: A close peep through

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SUMMARY The human body harbors approximately 10^{14} cells belonging to a diverse group of microorganisms. Bacteria outnumbers protozoa, fungi and viruses inhabiting our gastrointestinal tract (GIT), commonly referred to as the "human gut microbiome". Dysbiosis occurs when the balanced relationship between the host and the gut microbiota is disrupted, altering the usual microbial population there. This increases the susceptibility of the host to pathogens, and chances of its morbidity. It is due to the fact that the gut microbiome plays an important role in human health; it influences the progression of conditions varying from colorectal cancer to GIT disorders linked with the nervous system, autoimmunity, metabolism and inheritance. A rare disease is a lethal and persistent condition affecting 2-3 people per 5,000 populaces. This review article intends to discuss such rare neurological, autoimmune, cardio-metabolic and genetic disorders of man, focusing on the fundamental mechanism that links them with their gut microbiome. Ten rare diseases, including Pediatric Crohn's disease (PCD), Lichen planus (LP), Hypophosphatasia (HPP), Discitis, Cogan's syndrome, Chancroid disease, Sennetsu fever, Acute cholecystitis (AC), Grave's disease (GD) and Tropical sprue (TS) stands to highlight as key examples, along with personalized therapeutics meant for them. This medicinal approach addresses the individual's genetic and genomic pathography, and tackles the illness with specific and effective treatments.

Keywords disorders, human gut microbiome, personalized therapeutics, rare diseases

1. Introduction

Microorganisms are ubiquitous, living in and on us. The human body is made up of distinct populations of billions of microorganisms that inhabit certain areas of the same, and execute various metabolic and immunological functions. Exclusively 25% of the human body is made up of human cells; the rest is of hundreds of species of bacteria, fungi, protozoa and viruses. The genome of all microbes is collectively known as the "microbiome". Microbes contribute an additional 2 million genes to the 20,000 genes already encoded by the human genome (*1*).

The balanced connection between the host and the microbiome maintains a healthy human body. The diversity of the microbiome has an overall influence on human health in various categories, and its malfunctioning or dysbiosis directs to ailment, the diversity of which is distinguished by the abundance and diversity of distinctive species of bacteria correlated with such diseases (*2*).

A microbial community constitutes from the time when a human being is first exposed to the environment. The human body has an extensive spectrum of areas for bacteria to infiltrate, and one of the most prominent is the mucous membrane, which is found throughout the body. The microbiome aids in the extraction of energy and nutrients from the food we eat, as well as impeding the colonization by pathogens (*1*).

This microbial community emerges from the birth process, and has a significant influence on health. The infant microbiome and its assembly are determined by maternal-offspring exchanges of microbiota. The human body attains the microflora that is present along the birth canal during parturition, whereas individuals born by Caesarian section have a distinct skin microbiome as compared to individuals who are born vaginally (*3*). It is considered better to be born through the normal routes that acquire a more beneficial microbiome. The origin of microbes in the intrauterine environment, complex genetic dynamics of transmission and their site-

specific colonization, and immune system development in the infant remain to be determined yet. The maternal microbiota and its metabolites transferred to the fetus play a key role in influencing infant immune responses. Immunoglobulin G (IgG) from breast milk in many mammalian species is delivered into the neonatal circulation through intestinal epithelium. It contains about 88% water, and 124 g/L macronutrients, including about 7% (60-70 g/L) carbohydrates, 1% (8-10 g/L) protein, and 3.8% (35-40 g/L) fat. Breast-fed infants have higher levels of fatty acid oxidation products (preference for fat metabolism) in comparison to formula-fed ones (*4*). Therefore, the best source of cellular energy and nutrients for the rapid growth of the brain in an infant is breast milk in the early growing months. Breast milk is a fundamental contribution to the gut microbiota. The most researched organisms of gut microbiome that are transferred *via* breast feeding include *Lactobacillus* spp., *Bifidobacterium* spp. and *Bacillus subtilis*. There are very few genetic variations in the *Lactobacillus* spp., while there are comparative phenotypic and genetic variants seen in *Bifidobacterium* spp.; both in infants and mothers (*5*). Breast feeding is also an excellent indicator of promising health, as it protects against infections through specific and non-specific immune factors, and demonstrates beneficial effects on the intestinal flora of an infant. Breast milk strengthens the immature immune system of the neonates, and amplifies their defences against foreign entities. Immunoglobulins (Ig) excreted in milk are IgG that protect mainly against enteric infections. Breast milk also contains anti-idiotypic antibodies capable of increasing infant antibody response. Newborns that have a defect in fetching maternal immunoglobulins from breast milk are therefore at high risk of systemic infectious diseases (*6*).

Ailments or infections can be amassed in two ways: exogenously acquired infections from an external source such as bacterium in the environment, and endogenously acquired infections produced by agents inside the body as a result of antibiotic therapy altering the microbiome. Diseases become rare or ultra-rare when there is a consequence on a relatively low number of individuals worldwide and are often caused by errors in DNA (genetic cause). Drugs, cognitive and emotional stress, diets high in protein, simplified sugars/refined carbohydrates, fat or fructose, chemotherapy, radiotherapy and intestinal infections are all probable factors of dysbiosis. To reduce the cause of dysbiosis, probiotics and fecal biotherapy play a crucial role. Fecal biotherapy or fecal transplantation and Probiotics are important in reducing the etiology of dysbiosis (*7,8*). The presence of viruses, fungus, and bacteria is used to interpret the microbial community, and if there is a low abundance and density of these communities, it is referred to as the dark matter of the human microbiome. These values can also be misinterpreted by the dysbiosis of the microbes (*9*).

There are so many different rare diseases that do not necessarily mean it is very rare to have a rare disease. Studying these rare diseases is given utmost importance, with research investigations revealing deeper insights into human body working mechanisms. This is because many rare diseases are caused by relatively simple and known mechanisms, and these can even reveal about the bases of relatively common diseases that occur. There are interconnections between microbiota imbalances or alterations, and certain disease manifestations. Changes in microbial compositions can cause diseases such as neurological and autoimmune disorders, besides cardio-metabolic and genetic diseases, as well as impacting an individual's behavior to even trigger behavioral disorders in some cases (*10*).

2. Human gut microbiome and its association with rare diseases

2.1. Pediatric Crohn's disease (PCD)

PCD is a rare, inflammatory disease of the intestinal wall or portions of the gastrointestinal system, primarily symptomized by severe, chronic inflammation. This ailment is thus categorized as a subtype of inflammatory bowel disease (IBD) induced by altered microbial populations and disruptive intestinal immune responses. PCD mainly prevails in children of age below 2-3 years, and impacts 2 in 1,00,000 children aged younger than 10 years. A peak preponderance of PCD is seen in children aged 10-14 years, and it is very common in major nations like Australia, Scotland, and UK (*11*). Epidemiological studies indicate that there is an incremental increase in the number of affected individuals in Europe and the USA (*11*). PCD is an autoimmune ailment in which the immune system targets its tissues, inducing metabolic alterations (*12*). It is accelerated by the inactivation of the mucosal immune response correlated to host genetics, microbial community alterations and environmental factors. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium (*13*). Reduced microbial multiplicity illustrates dysbiosis in the gut microbiota, and those with PCD have lower levels of *F. prausnitzii* and higher levels of *Escherichia coli* (*14*). Butyrate, the end product of intestinal bacterial fermentation of primarily non-digestible carbohydrates such as resistant starch, is absorbed by the colon cells through transport pathways. There, the butyrate is metabolized to generate energy that sustains the integrity and health of the colon cells. Butyrate, being a substrate of fatty acid metabolism generates ATP in our body, furthermore maintaining the lining of the colon. Decreasing levels of *F. prausnitzii* can reduce ATP intake in these cells, resulting in a loss of anti-inflammatory function and a weakening of the ability to fight infections (*15*). The affected individuals may forfeit digestion and absorption functionalities. Treatment of PCD involves restoration of

the mucosal lining, wherein "exclusive enteral nutrition (EEN)", formulated with polymeric nutrition, is fed to the affected individuals. EEN aids in the diagnosis of intestinal microbiota and faecal microbiota alterations. It incites the alterations in gut microbiota, which leads to a reduction in inflammation (*16*).

2.2. Lichen planus (LP)

Lesions on the skin, urogenital tissue and nails, and eyes are observed in LP-affected individuals. Oral bacteria not only maintain oral hygiene, but also influence the development of many oral diseases. *Capnocytophaga sputigena*, *Eikenella corrodens*, *Lactobacillus crispatus*, *Mobiluncus curtisii*, *Neisseria mucosa*, *Prevotella bivia*, *Prevotella intermedia*, and *Streptococcus agalactiae* are the microbes that tend to infect the oral mucosa and are found in oral lesions. The metabolic disturbance or the imbalance in the metabolic process is the main cause in the colonization and proliferation of fungal species that affect the epithelial cells in lesions and the nutrient uptake and release of cytokines are hindered causing the disease condition (*17*).

Oral lichen planus (OLP) could either accompany cutaneous lesions in the oral cavity, or precede them. These lesions are designated as lichenoid mucositis or lichenoid lesions. Stress and anxiety are known to be substantial factors in inciting this condition. A combination of drugs and other foreign bodies may also elicit a host response to induce these lesions (*18*).

OLP lesions manifest themselves in a variety of clinical forms namely, reticular, atrophic or erythematous type, erosive or ulcerative, plaque or hypertrophic form, and bullous form. Reticular OLP is a common type of condition where it manifests in buccal mucosa and other sites such as the tongue, gingiva, and lips. It is characterized by numerous interlacing white keratolytic lines striae, called Wickham's striae. This occurs with mild symptoms or may be asymptomatic. Atrophic or erythematous form occurs as a red patch in conjunction with reticular striae (white straie) and with an erosive variant. Symptoms like burning sensation, discomfort and lesions are manifested on gingival, producing a pattern called desquamative gingivitis, also known as pemphigus vulgaris, cicatricial pemphigoid or epidermolysis bullosa. The erosive or ulcerative form manifests with an ulcer covered with pseudomembranous exudates associated with reticular striae and also erythematous patches. White raised or flat plaques may occur as a variant of lesion usually on the tongue and buccal mucosa. This may occur in conjunction with white striae and irregular smooth plaques, often involving one or more than one affected area. OLP also manifests as patchy areas of reactive melanosis due to stimulation of melanocytes by inflammatory cells (*19*).

Another variant of this disease is the "lichen planus pigmentosus-inversus" which is characterized by spots on cervical, axillary and popliteal regions. This disease-inducing immune system generates an autoinflammatory response in the oral cavity, hair, and nails (*20,21*).

Lichen sclerosus is another form of autoimmune condition and is triggered by *Borrelia burgdorferi* which is the evidential cause of Lyme disease. Salivary mycobiome (*Candida* spp., *Torulopsis glabrata*, *S. cerevisiae*, *Aspergillu*s spp., *Erysiphe* spp.) dysbiosis leads to a shift in oral bacteriome associated with the disease (*21*). Some of the other species of fungi involved are *Epidermophyton inguinale*, *Trichophyton gypseum*, *T. interdigitale*, *T. purpureum*, *Cryptococcus hominis*, and *Oidium pulmoneum* (*22*).

Some of the viruses also involved in this disease are herpes simplex virus 1 (HSV-1), Epstein Barr virus (EBV), human papillomavirus (HPV), hepatitis virus and cytomegalovirus (CMV) (*23,24*).

2.3. Hypophosphatasia (HPP)

HPP is a disease of bone mineralization. Patients with severe instances, with a rare presentation of one in every 100,000 individuals, have fragile bones that are frequently fractured and deformed. The occurrence of persistent pain, as well as the early loss of teeth and fractures, is common (*25*).

This condition is caused by a gene mutation that inhibits the body from producing the enzyme alkaline phosphatase. This enzyme regulates the formation of a chemical called pyrophosphate, which is present in blood and urine, and it stops the key mineral in our bones from growing. Mineralization does not occur in the body due to pyrophosphate build up, resulting in the disease condition of HPP. Intestinal alkaline phosphatase is also helpful in improving and maintaining appropriate gut barrier function, and serves as a gut mucosal defense mechanism. It is influenced significantly by bacterial exposure (*26*).

Individuals who have HPP also have periodontitis, which is a polymicrobial infectious disorder that affects the teeth enamel. Periodontitis is caused by a shift in the microbial ecology of the teeth. *Porphyromonas* species are found in the subgingival cavity, resulting in the deterioration in the arrangement of teeth in people suffering from this disease (*27*). *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are periodontopathic bacteria that play a significant role in the pathogenesis of the disease. Among these three pathogens, *P. gingivalis* is most commonly seen in patients with periodontitis. The bacterial flora differs at both subgingival and supragingival levels, and so do the metabolites produced by the pathogens. Plaques occur at the supragingival level as a result of the bacterium *Streptococcus mutans* producing lactic acid, which directly causes dental caries by decreasing the pH of enamel. In the progression of periodontal disease, the periodontal pathogen *Fusobacterium nucleatum* and

P. gingivalis produce short-chain fatty acids (SCFA), including butyric acid, as metabolites. Butyric acid, also known as butyrate, is known to function intrusively on periodontal tissues. Increased butyrate concentrations have been associated with inflammatory disease in the brain, and cause apoptosis in human gingival fibroblasts following long-term exposure to butyrate (*28,29*).

2.4. Discitis (diskitis)

Discitis (diskitis) is an inflammation or infection in the spine. These are unique to the intervertebral discs of the spine. Pressure on the disc causes inflammation or swelling, which finally leads to discomfort or agony. There are two forms of discitis, namely intervening discitis and spontaneous discitis. In the intervening discitis, diagnostic or therapeutic procedures can induce infection at the location and cause inflammation, whereas spontaneous discitis is caused by microbial invasion through the blood circulation (*30*).

If the intervertebral discs are not furnished with blood, microorganisms may intrude and cause infection. These microorganisms can enter the bloodstream by any route, including the urinary tract, the respiratory tract, the pelvic region, and the gums. Younger adults are more susceptible to this infection, with rare occurrence in the older people. It is common only in people with diabetes (*31*).

Spondylodiscitis (SD) is a form of spontaneous discitis in which the inflammation in the intervertebral discs or nearby vertebral bodies is caused by bacterial interventions. There are several forms of SD, including pyogenic (bacterial), granulomatous (fungi) and parasitic (*Echinococcus* spp.) (*32*).

Staphylococcus aureus, *Streptococcus* spp. and *Enterococcus* spp. induce pyogenic SD by releasing proteolytic enzymes that disintegrate the discs. These bacteria penetrate the metaphyseal arcades and deposit on the cartilaginous plates, which allow them to live and release the enzyme. The infection can also cause pus to accumulate around the spinal cord (paravertebral abscess), and fractures can happen, eventually leading to meningitis, spinal epidural abscess, and neurological impairment (*33,34*).

Some of the other infection forms are vertebral osteomyelitis, spondylitis, epidural empyema, epidural phlegmon and diabetic foot ulcerations (DFU) (*35*). The opportunistic pathogen *S. aureus* causes DFU by colonizing the wound in multi-layers and forming biofilm. This pathogen is also commonly responsible for spinal osteomyelitis/vertebral osteomyelitis. The wound will aggravate as glycemic control deteriorates, causing the immune system to fail to react (*36,37*).

Septic arthritis is rare, with morbidity rates of 40-50 percent and death rates of 10-20%. It is caused by microbial entry through the circulation, which creates joint pain (*38)*.

2.5. Cogan's syndrome

DG Cogan reported the classic form of the Cogan's syndrome disease in 1945. It is a conglomeration triad of non-syphilitic interstitial keratitis, vestibuloauditory illness, and respiratory tract symptoms (*39*). It is also a cause of vasculitis (autoimmune vasculitis) (*40*).

Cogan's syndrome is a very rare autoimmune disorder in which autoantibodies cause tissue damage in portions of the ear, including endothelial cells and inner sensory epithelial cells. Cogan's syndrome symptoms are comparable to Meniere's syndrome, with the abrupt onset of nausea, vertigo, vomiting and hearing loss. It is characterized by continual inflammation of the frontal portion of the eye, particularly referring to cornea (*41*). The pathogenesis of ocular infections in Cogan's syndrome includes *Chlamydia psittaci* (Psittacosis), *C. pneumoniae* (pneumonia), and *Chlamydia trachomatis* (trachoma) (*42,43*). *Chlamydia psittaci* causes myocarditis and valvular lesions (*44,45*).

The ocular condition is characterized by interstitial keratitis which causes vision impairment, conjunctivitis and inflammation around the eye, as well as an increase in light sensitivity. Interstitial keratitis can coexist with syphilitic disease, which is often caused by a combination of bacteria and viruses associated with systemic vasculitides (*46,47*). Viruses such as Herpes simplex virus (HSV), Epstein Barr virus (EBV) and Rubulavirus cause viral infections presented in corneal disease. Corneal inflammation has been linked to a number of diseases, including Polyarteritis nodosa, Wegener granulomatosis and Rheumatoid arthritis. Rubulavirus causes lacrimal gland inflammation that involves the cornea, resulting in "blue eye disease". Rubulavirus also causes another sickness known as Waardenburg syndrome, which is a rare, genetically inherited illness characterized by hearing loss and a pigmentation defect that causes blue and brown eye pigmentations in the eye. This blue pigmentation is induced by a member of the *Orthorubulavirus* viral species. The virus enters the body *via* exposures, and is hypothesized to replicate in the nasal mucosa and tonsils before spreading to the brain and lungs (*48*).

2.6. Chancroid disease

Chancroid disease is a rare infection caused by the Gram negative, facultatively anaerobic bacterium *Haemophilus ducreyi,* and is acquired through sexual contact. It is a genital ulcer condition, which has been linked to human immunodeficiency virus (HIV) transmission (*49*). *H. ducreyi* penetrates the skin through a breach in the mucosa, where it produces a toxin (cytocidal distending toxin) that destroys individual cells in the infected region, while also triggering a local inflammatory reaction that causes necrosis and leads to ulcer aggravation. Within the first week of onset, infected individuals will develop

papules, which will subsequently develop into pustules, which are more prevalent in males than females, and inevitably ulcers, which can be excruciating (*50*). Lymph node inflammation (lymphadenitis) also occurs, resulting in a tiny lump that causes severe pain. These nodes rupture in some cases, resulting in abscesses (*51*).

This disease also includes the forms of syphilis, genital herpes and donovanosis (granuloma inguinale) (*52*). These diseases are solely caused in humans, with no intermediate hosts, and it is extremely infectious and contagious. Donovanosis is caused by the bacterium *Klebsiella granulomatis*, which causes scarring and necrosis in the afflicted region. Ulcers most commonly develop on the labia and internal to the vaginal or on cervix in women, and on the penile foreskin in men (*53,54*). Chanchroidal ulcers are greyish in appearance, which differentiate it from ulcers caused by donovanosis and syphilis (*55*).

2.7. Sennetsu fever

Sennetsu fever is a fever-like disease caused by *Ehrlichia sennetsu*, a member of the *Ehrlichia* bacteria family. It is a rare disease condition that belongs to the Human Ehrilichioses category of diseases. Human monocytic ehrlichiosis (HME) caused by *Ehrlichia chaffensis* and Human Granulocytic ehrlichiosis (HGE) caused by Anaplasma phagocytophilium are two related types of the ehrlichial infections. Individuals who are infected or who have contact with infected people can develop symptoms such as high fever, headaches, myalgia, nausea, vomiting, anorexia and insomnia (*56*).

The consumption of raw fish is thought to be the primary source of this fever, which will eventually progress to the disease state. The encystment of larval nematode can be found in raw or undercooked fish, and it is passed to humans *via* outer membrane proteins of the bacteria attached to the host cell, where it activates an inflammatory response by associating with capillaries and arteries (*57*). Transglutaminase activity initiates endocytosis by allowing phagocytes to enter the host. Endocytosis is activated in response to pathogen entry, and creates a vacuole for pathogen survival in the host cell. It will inhibit macrophage entry and cause the macrophage to dissolve and thus cause enlargement of liver, spleen and lymph nodes (*58*).

2.8. Acute cholecystitis (AC)

Gall bladder plays an imperative role in storage and concentration of bile juice secreted by the liver, but development of gallstones near the cystic duct leading to severe gallbladder inflammation is commonly referred to as AC (*59*). Predominantly the incidents of AC occur due to impediment of the cystic duct by gallstones, or in few cases, it can be due to aggregation of gallbladder sludge. Protracted obstruction of the cystic duct leads to severe inflammatory responses in gallbladder, which in turn, triggers gut-wrenching infections and formation of biliary sludge or gallbladder sludge, *i.e*. aggregation of sedimented particulate matter consisting of remains of bile juice, calcium salts, crystals of cholesterol and pigmented calcium bilirubinate (*60,61*).

This gastrointestinal disorder closely interacts and houses the secondary bacterial infections from the enteric microbes, as well as contributes towards the proliferation of opportunistic microbes (*62*). According to clinical researches conducted worldwide, enteric Gram-negative anaerobic gut microbiome interactions present in the biliary system points to an imperative association, which help us in understanding the patho-physiological conditions of the patients affected with AC, comprising of the growth of *Clostridium perfringens*, *Citrobacter freundii*, *Klebsiella* spp., *Escherichia coli* and *Enterobacter cloacae* (*63*).

Consistently higher populations of enteric gut microflora near the biliary region of the patients affected by AC play a significant role in the production of bile juice and to the development of gallstones (*60*). Traces of elevated similarity have been found between the microflora present in the duodenum with the biliary microbiome, which actively participates in the polymerization and oxidation of bile juice, leading to the development of gallstones (*64*).

The secretion of the enzyme cholecystokinin (CCK), which plays a considerably significant role in the storage and concentration of bile juice in the gallbladder, is produced by the immunological stimulation of buccal microbial population. Development and progression of oxidative stress conditions, inducing free-radical reactions in the bladder mucosal cells, stimulate the process of gallstone formation, and impedes the physiological functioning of the gallbladder (*65*).

Other pathogenic microbes like the spiral-shaped *Helicobacter pylori* perpetrates in elevation of clinical complexities like up-regulation of urease which stimulates the precipitation of calcium ions during the development of gallstones, leading to the progression of chronic and AC. Furthermore, *H. pylori* elevates the inflammatory responses produced in the patients suffering from AC, with traces of interleukins (ILs) also detected in them, namely classes I and VI, along with tumor necrosis factor (TNF)-α, eliciting such immunological responses (*66*). Immunocompromised patients with several co-morbidities and other complexities tend to develop enriching bacteriological infections which can be lethal (*63*).

Nowadays, advanced diagnostic techniques involve ultrasonography (USG), computerised tomographic (CT) scan or by hepatobiliary iminodiacetic acid (HIDA) test, which can easily diagnose and detect any abnormalities in the biliary duct. Primarily the treatment stratagem involves surgical procedures like cholecystectomy *i.e*. an invasive surgical protocol

to remove the malfunctioning gallbladder with the gallstone (*63*). In certain minor cases, endoscopic retrograde cholangiopancreatography (ERCP) can be used to eliminate the gall stones obstructing the cystic duct. Administration of antibiotics and painkillers are subject to variability, depending on the degree of microbial infection or inflammatory pain (*67*).

2.9. Grave's disease (GD)

The thyroid gland plays a significant role in regulating our metabolic rates *i.e*. synchronizing the rate at which our body utilizes the energy. However, during hypersecretion of thyroid hormone (thyroxine), it can cause an autoimmune disorder termed as GD or hyperthyroidism (*68*).

As an autoimmune disorder, the immune system tries to attack thyroid gland for the over or hypo-secretion of thyroxine (*69*). The primary immunological cause for the hypersecretion of thyroid glands is the attachment of an immunoglobulin molecule produced as an autoimmune response, termed as thyroid-stimulating immunoglobulin (TSI) which basically substitutes the natural thyroid stimulating hormone (TSH) (*70*). A person with pre-compromised immunity or undergoing an existing autoimmune disease like vitiligo, rheumatoid arthritis, lupus or celiac disease have a greater chance of developing this condition (*71*).

According to scientific reports and research conducted worldwide, the gut microflora population elicits promising insights on diagnosis, patho-physiology and treatment of Grave's disease. Following next generation sequencing (NGS) and characterization, varied gastrointestinal microflora populations were reported from the faecal samples of patients and healthy controls. A peculiar microfloral trend was observed during pathological diagnosis and research, where species of *Prevotella, Bacilli*, Lactobacillales, *Veillonella* and *Megamonas* outnumbered in samples of an infected individual, whereas species of *Alistipes*, *Ruminococcus* and Rikenellaceae were found to decline in the infected sample when contrasted to a healthy control sample (*72*). This particular scientific finding furnished a preliminary insight that the noticeable alterations in the gut microflora population between healthy controls and infected patients had certain promising links with the occurrence and progression of the disease (*72*).

Antibiotics play an imperative role in decreasing a bacteriological infection but instances of using it can affect the gut microflora and remarkably which can alter the levels of blood pressure, progressing towards the development of hypertension, an essential characteristic of GD. It was studied and observed that bacteria belonging to *Bacteroidetes* and *Firmicutes* have an interrelation with elevated blood pressure levels in the body, which directly relates to hypertension (*73*).

As reported by dry and wet lab research, *Lactobacillus* and *Bifidobacterium* stimulate the production of high levels of cross-reactive immunogenic responses which escalates the autoimmune condition elicited by patients (*74*).

The levels of TSH in the blood, preferably higher, are an indicative clinical marker for an efficiently-functioning thyroid gland. These healthy levels can be easily measured in the bloodstream by performing simple blood tests as a diagnostic measure for adequate prior treatment. Alternatively, USG or CT scans can be used to visualise the abnormalities in the thyroid gland, such as certain protrusions or enlargement. Treatment approaches encompasses clinical methods to reduce the hypersecretion of the thyroid gland, which can be achieved by employing anti-thyroid drugs or radioactive iodine. In certain grave conditions, the thyroid gland might have to be surgically removed or destroyed by using radioactive iodine to reduce the ill-effects of hypersecretion of the gland (*75*).

2.10. Tropical sprue (TS)

TS, a rare digestive disease of unknown etiology, mainly affects the small intestine of tribes living in tropical or temperate regions such as the Caribbean Islands, India, South Africa, and Southeast Asia. TS are characterized by the intestinal malabsorption of nutrients and minerals, besides persistent diarrheal conditions in the body (*76*).

Clinical findings and pathophysiological studies confirm the correlation between gastrointestinal microflora and TS infections (*77*). Pathogenesis of TS is peculiar, postulating that the mucosal injury of the jejunum and ileum leads to bacterial overgrowth in the small intestine (*78*). Subsequently, folate and vitamin B12 deficiencies cause the mucosal lining to become more susceptible to damage. Increased secretions of enterotoxins by the gastrointestinal bacterial population (*Klebsiella*, *E coli* and *Enterobacter*) mediate the infection (*79,80*). Bacterial colonization elicits unregulated production of a gut hormone (enteroglucagon) and motilin peptide by endocrinocytes of the proximal small bowel (*81*).

The clinical findings of TS show dyspepsia, anorexia, weight loss and multiple nutritional deficiencies that arise from dysfunctional fat digestion at the intestinal level. Insufficient pancreatic lipase, defective mucosal membrane and impaired intestinal transport system causes disruptions in the bacterial flora, and influences the development of gastrointestinal infections in TS (*82*).

Absorption and digestion of long chains of fatty acids is a principal function of the small intestine by bile and pancreatic juice. Digestive juice breaks the fats into simpler compounds and absorbs nutrients in the duodenum. Triacylglycerides (TAGs) upon emulsification and hydrolysis forms L-glycerol and free fatty acids (FFAs). Later, enterocytes utilize these FFAs for the biosynthesis of fats (*83*). Thus, it is apparent

that the gut microbiota is affected by a diverse range of dietary TAGs, and could play a role in the pathogenesis of TS (*84*).

The combined therapy of antibiotics (tetracycline or ampicillin) along with folic acid and vitamin B12 supplementation improves the symptoms of the infection, and reduces the bacterial population. Anti-diarrheal drugs may control the severity of diarrhea, but D-xylose malabsorption and water and electrolyte secretory defects persist, despite prolonged therapy owing to the damaged mucosal membrane (*80*).

3. Personalized medicine (PM) in rare diseases

Human genome is very unique; it varies from person to person, and people show differential responses to different treatments. Personalized medicine (PM) streamlines medication based on a patient's genetic make-up, by focusing on molecular profiling, medical imaging, clinical statistics and data (*85*). The Father of the PM is Archibald E. Garrod (1857-1936), who described the ubiquity of the individual variations (*86*). PM is also termed ‘precision medicine' as it customizes the diagnostics, drug/or products based on the patient's response to disease and its severity, in order to offer the optimal therapeutics. With the advent of technology, the dawn of precision medicine has revolutionized the medical sector. Scientific advancement has paved the way to new recognition of the treatment and management of complications in diseases at personal levels. PM is thus a welcome deviation from "one-size-fits-for-all" medical approach with different applications such as in oncology, cardiology, autoimmune disorders, nutrition and rare diseases (*87,88*).

According to a recent report by the World Health Organization (WHO), there are about 7,000 rare diseases that affect 7% of the total population with no appropriate treatments for approximately 95% of such rare diseases. Patients with undiagnosed and unknown etiology of the diseases often are overlooked by an uncertain and unpredictable journey, referred to as a "diagnostic odyssey" (*89*). PM is a medical tool to end this diagnostic odyssey by giving way to new therapeutics.

Mutations in genotypic or phenotypic traits influence the severity and reactivity of the diseases to a particular potential therapy. The cellular response and the behavior of particular mutations to various therapeutic options vary in great lengths among different individuals when contributing to certain drug responses, as well as on the patho-physiology of the disease they are struggling with. The concepts and definitions of PM are based on the patient's genomics, epigenomics, proteomics, metabolomics, lipidomics and other data relevant to lifestyle. Rarity of the diseases creates ultimatums when citing fundings, affordability of treatments and diagnostics, computational analysis and clinical trials. Therefore, the approach of precision medicine is an evolution gateway in healthcare and disease management. Perspective of precision medicine is to intercept disease prevention and treatment as per the variability of genes, environment and lifestyle of an affected individual (*90*).

Precision therapeutics aims to escalate the efficacy and diminish the toxicity of the drug in healthcare. PM is not only an individualized drug, but a medicine that incorporates both standardization and individualization (*91*). Thus, harnessing PM in the treatment of rare disease could prove effective, and employ alternative budding therapies and diagnostics for rare diseases in the coming few years. However, there are many challenges in the future that we might have to deal with, such as lack of therapeutic efficacy for various ailments, high cost of pharmacological diagnosis and its clinical trials, wide comprehension of genetic diversity, broadening the medical research projects and better implementation of computational analysis. Despite the use of complex technologies and large-scale whole-genome sequencing (WGS), it is still difficult to understand the adaptive nature of the biological system regarding changes or responses to drugs (*92*). Thus, personalized therapeutics need implementation at a holistic level to overcome many of these barriers in the development of a new medical era.

4. Conclusion

The development and progression of a disease depends upon multiple facets of phenotypic, genetic and epigenetic factors which are designated and demarcated by distinct geographical regions. Deriving a concrete precision and clarity about a particular ailment can be ambiguous, and can depend upon manifold surveys conducted on interconnected flora, fauna, humans, microbes and their subpopulations associated with it. The dearth of concurrent facts and scientific research in a particular domain of disease biology might affect its diagnosis, therapeutic approaches and future research.

Rare diseases torment millions of lives belonging to all age groups globally, which could prove life-threatening. Specific rare diseases differ in multi-facets with distressing shifts. Most of these diseases arise due to genetic mutations and are inherited, which goes on undiagnosed for several years or maybe generations. A number of rare diseases reported till date varies by a margin and newly identified illnesses are reported weekly. In 2017, National Policy for Treatment of Rare Diseases, India, estimated that there were around 5,000 to 8,000 rare diseases, with 450 of these recorded in India, affecting 72 million - 96 million Indians, with the majority being children.

Rare diseases belong to such a field in biology which has lack of concurrent evidence on common medical symptoms, disease development, genetic history, pathogenesis, and transmission routes. Rare diseases have been customarily interpreted as the set of ailments

which only affects a fewer section of the population, and if the clinical manifestations are not dealt with adequate medical attention, then it can be lethal. The prime complication and setbacks faced by the medical and clinical research fraternity is the ardousity in identifying and comprehending the odd patho-physiological manifestations for the rare pathologies. To some extent, recent advancements in the sectors of clinical research, biosciences and medicine have paved a path in smooth diagnosis, prognosis and treatment for the patients, but still we require full-proof solutions to the unsolved clinical challenges raised since time immemorial for the treatment of rare diseases.

Standing today, the predominant challenge lies in adequate identification of the rare diseases and fabricating a specific diagnostic and therapeutic approach to mitigate the shortcomings, but this identification is shrouded with an array of coinciding definitions, theories, concepts and metadata which influence the disease identification. Corroborating such a plethora of juxtaposed concepts with computational aids to segregate the subtypes of rare diseases based on genetic, pathogenic and epigenetic factors is something that has been done and is still under process. Worldwide researches have been conducted to clinically computerise and conglomerate such meta-information to aid in developing a holistic and in-depth knowledge about the rare diseases.

The human body is a compatible host for the microbiota that it inhabits; the human microbiome plays an imperative role in interconnecting the missing links between progression of an ailment and our body. It is scientifically proven that human microbiota is of prime importance when it impacts our metabolism, physiological behaviour, disease responses, immunological manifestations and other physio-chemical manifolds of human anatomy. Predominantly our body houses a substantially colossal load of microbes in the gastrointestinal and urinary tracts, whose imbalance in our body can lead to several irritable discomforts and to a certain extent, it escalates into development of a clinical ailment requiring immediate medical attention. The diversity, utility and complexity of the microbiota found in a human being is a subject of debate, the factors which can determine and characterize the nature of microbiome population remain deluded and varies from person to person, lifestyle, genetic or epigenetic conditions, and more often intervene in all life processes.

Advancements in science and technology in recent years have significantly unlocked the heap of data, yielding insights into the role of the human gut microbiome in existing models of rare diseases and clinical assortments. Understanding the nature of gut microbiota, and their dynamic interplay with host and other agents have enabled scientists and researchers to devise new diagnostic techniques and interventional strategies. Interestingly, at least 5-10% rare diseases are linked with human gut microbiota. Gut complications of secluded populace are often exacerbated by alterations in gut microbiome population, leading to occurrence of the disease. The gut microbiota is a pivotal agent of body homeostasis, barrier function, and development of immunity, nutritional responses and metabolism processes in humans. The impact of commensal microorganisms is not limited to the gastrointestinal tract, but rather encompasses all organs of the human body. With the abundance of metagenomic data and increasing research on gut microbes, one can precisely claim the association between commensal bacterium and pathogenesis of the diseases. For example, a myriad of investigations have reported changes in the gut microbiota not only during obesity, diabetes, kidney and liver problems but also cancer, and even some rare neurodegenerative diseases. In addition to regulation of infection and commensal spread, microbiome-mediated-immunity are implicated in a variety of ‘non-communicable' gastrointestinal diseases, as well as extra-intestinal disorders. Emerging evidence establishes that the microbiomes of extra-intestinal mucosal surfaces provide niche-specific functions that aid in the pathogenesis of certain underlying disorders.

The aim of this review is to summarize the clinical findings and research analysis by introducing the reader to emerging challenges, potential trends and current directions in microbiome research. It is challenging to state a correlation between a local microbe and disease before showing the implication of bacterium on the onset of the particular disease. For numerous rare diseases, basic knowledge such as the etiology of the disease, patho-physiology and epidemiological data hinders the prevention and treatment of the disease. Furthermore, the association between the human microbiota with the host is far more complex than observed with tremendous data till date. Hence, emendation of experimental designs and regular diagnosis to study the disease from its progression to latent stages is the call of the hour.

The idea behind this discussion is to delineate the effective therapeutics to overcome the limitations of existing treatments and identify the budding interventional approaches. Human microbiome research is a rapidly accelerating field with the preliminary studies increasing exponentially, and more discoveries continue to be made in the frontiers on the role of gut microbiome in human wellness. With the dawn of this new era, human microbiome research is becoming a more transdisciplinary field with a colossal range of applications and strategies for understanding it. Anticipation of mathematical and computational tools to understand the microbiome has reformed the status of diagnosis, prognosis, prophylaxis and prevention of human diseases in ongoing research. In-order to fill the current vents, translation and transplantation of experimental evidence through consistent use of pre/or probiotic matrix to protect our gut from pathogens and versatile methods can be done to assess changes in

health outcomes. As researchers learn more about the human microbiome and develop more-robust techniques for probing to leverage our insights, a plethora of new diagnostic tools and interventional methods that could revolutionize medicine and treatment of rare diseases would come to the limelight.

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Mini-Review

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Expanded newborn bloodspot screening: developed country examples and what can be done in Turkey

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SUMMARY Bloodspot screening in newborns is an exemplary public health intervention as it is essential secondary prevention with proven efficacy and benefit for the early diagnosis and prompt treatment of rare diseases. In this mini review, newborn bloodspot screening (NBS) programs of 12 countries were examined in terms of the extent of diseases/disorders screened to form recommendations for Turkey's expanded newborn screening program. Essentially, Turkey and 11 selected countries' official policies/national programs or strategies in terms of newborn screening and the number of diseases/conditions screened were examined. The current status of spinal muscular atrophy (SMA) screening was also checked through the SMA NBS Alliance. In addition, WHO and EURORDIS guidelines for newborn screening were also reviewed. On the Pubmed database, following the search strategy "((newborn screening[Title/Abstract]) OR (newborn screening program[Title/Abstract])) OR (newborn blood spot screening[Title/Abstract])" in the PubMed database from 1 January 2008 to 1 December 2021. Diseases that will be recommended to be included in the Turkish national newborn bloodspot screening program will be presented by evaluating the updated criteria of Wilson and Jungner by constructing international comparisons. The number of diseases/disorders screened by the inspected 12 countries is eminently variable and ranges from 5 in Turkey to 51 in New York, United States of America (USA). Acknowledging the programs of other countries, it is evident that Turkey must advance its program by evaluating the epidemiological data in Turkey, the health workforce, and infrastructure while relying on the updated screening criteria. The newborn bloodspot screening program should be expanded based on the cost estimates and implemented starting with pilot applications and the diseases/disorders that are deemed appropriate should be included in the national program.

Keywords rare diseases, neonatal screening, newborn screening, dried blood spots, secondary prevention

1. Introduction

Rare diseases are a group of diseases, most of which are genetically based, chronic, mostly life-threatening, a cause of severe morbidity, and have an onset at a younger age compared to other chronic diseases (*1,2*). While at least 3–4 new rare diseases are being identified annually (*3*), there are more than 6,000 rare diseases globally identified (*4*). To include a disease in the rare disease group, frequency criteria are used corresponding to countries and regions. While diseases that affect less than 1 in 2,000 people are described as "rare diseases" in the European Union (EU) (*5*) and Turkey (*3*). It is estimated that there are 5 million individuals with rare diseases in Turkey (*3*) and 350 million globally (*6*).

Rare diseases are recognized as a serious public health issue worldwide (*7,8*). Research, diagnosis, and treatment of these diseases are exceedingly challenging and pricey. In the early stages of the disease, some can have no symptoms, are misunderstood, or confused with other diseases. Due to the lack of convenient treatment options, these diseases are seen as health orphans because they have been neglected for years (*9*).

2. As a secondary prevention: bloodspot screening in newborns and expanding of the screened disease/condition(s) list

Bloodspot screening in newborns is an exemplary public health intervention as it is an essential secondary prevention with proven efficacy and benefit for the early diagnosis and prompt treatment of rare diseases (*10,11*).

However, over time, in addition to its benefits, it has become controversial due to the cost, clinical benefit, and violation of ethical values for the diseases to be included in a screening program (*12,13*). An expert group for EU member states has stated that newborn screening should be evaluated within the framework of a common policy within the Union. Experts expressed their opinions in the following: governance of neonatal screening; criteria to evaluate whether a screening program should be performed; criteria on how a screening program should be performed; informed consent; blood spot sampling; laboratory procedures; blood spot storage; communication of positive results; confirmation of diagnosis and treatment; communication of unintended findings; quality assurance of laboratory results; screening program evaluation; epidemiological evaluation; and features of disorders, which might be considered in the gradual expansion of NBS in EU. They also created a proposed model of a decision-making matrix for an appropriate and standard policy (*14*). Expanded newborn screening programs have been initiated in the world, especially in the European Region, North America, and Australia. For this reason, this article establishes recommendations for the expansion of the current newborn blood spot screening program in Turkey, based on national data, by examining international examples.

2.1. Current status in the number of disease/condition(s) screened in newborns internationally and some countries' policy

For this mini narrative review, essentially, Turkey and 11 selected countries' official policies in terms of newborn screening and the number of diseases/conditions screened were examined. The current status of SMA screnning was also checked through the SMA NBS Alliance. In addition, WHO and EURORDIS guidelines for newborn screening were also reviewed. On the Pubmed database, following with the search strategy "((newborn screening[Title/Abstract]) OR (newborn screening program[Title/Abstract])) OR (newborn blood spot screening[Title/Abstract])" in the "PubMed" database from 1 January 2008 to 1 December 2021. A total of 2,755 articles which was the full texts accessible were evaluated, and after evaluation of the abstracts and texts, 17 articles which reflected the opinions of international consensus' and assessments, and articles examining current policies and situations of countries where official current policy documents were not available were included. Studies on diseases/conditions not currently included in the national screening program were excluded. The Wilson and Jungner criteria serve as a guide for determining whether a disease is a suitable candidate for population-wide screening (*15*) and has been used for years (*16*). However, today, because of advancements in medicine, a demand for an update has arisen. In 2008 and 2018, two different groups of researchers identified new principles (*16,17*).

Accordingly, 12 criteria were listed under 3 headlines: disease/condition, test/intervention, and program/system principles (*16*). Diseases that will be recommended to be included in the Turkish national newborn blood spot screening program will be presented by evaluating the updated criteria of Wilson and Jungner by constructing international comparisons.

The national newborn blood spot screening programs of 12 countries (Australia, Canada, Germany, Denmark, United Kingdom (UK), Israel, Italy, the Netherlands, New Zealand, Norway, Turkey, and United States of America (USA) were examined.

2.2. Current situation in the number of diseases/conditions screened in 12 countries

The number of diseases/disorders screened by the 12 countries is highly variable and ranges from 5–51. The number of diseases/conditions screened are as follows: 32 in Australia (*18*), 25 in Ontario, Canada (*19*), 19 in Germany (*20-22*) 19 in Denmark (*22,23*), 9 in the UK (*24*), 12 in Israel (*25*), 40 in Italy (*26*), 25 in the Netherlands (*27*), 26 in New Zealand (*28*), 26 in Norway (*29*), 5 in Turkey (*30*) and 51 in the New York, USA (*31*).

The most frequently screened diseases in the 12 countries examined are as follows: phenylketonuria (PKU) and congenital hypothyroidism (n = 12); cystic fibrosis, glutaric acidemia type I, maple syrup urine disease, and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency (n = 11); isovaleric acidemia and very-long-chain acyl-CoA dehydrogenase deficiency (VLCAD) (n = 10); congenital adrenal hyperplasia, biotinidase deficiency, tyrosinemia type 1, methylmalonic acidurias, propionic acidaemia and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) (n = 9); severe common immun deficiency (n = 8); galactosemia, carnitine palmitoyltransferase-I (CPT-I) deficiency and homocystinuria and trifunctional protein (TFP) deficiency (n = 7); (multiple) holocarboxylase synthetase deficiency homocystinuria, carnitine acylcarnitine translocase (CACT) deficiency and carnitine palmitoyltransferase-II (CPT-II) deficiency (n = 6). Additionally, 33 diseases/disorders are screened in 1, 2, 3, 4, or 5 countries (Table 1, Online Data, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=100*).

3. Differences between countries and international standards

When the newborn blood spot screening programs of these 12 countries is examined in terms of the number of diseases/disorders screened, the New York, USA ranks first with 55 diseases/conditions, Turkey ranks last with 5 diseases/disorders followed by the UK with 9 diseases/disorders. Additionally, among the examined countries, Italy ranks first with 40 diseases/disorders

screened in the member countries of the World Health Organization Regional Office for Europe. However, New York, USA has the highest number of screened diseases/disorders with 54 diseases/disorders among the examined countries. The disease, commonly screened for in all of the examined countries, is PKU. The striking finding is that Turkey is the only country among these 12 countries that does not screen for glutaric acidemia type 1, maple syrup disease, and MCAD deficiency, and also one of only two countries that do not screen for isovaleric acidaemia and VLCAD deficiency. The prevalence of consanguineous marriages in Turkey is around 25% (*32*) where consanguineous marriages are a risk factor for rare diseases. Therefore, there is a demand for an expanded newborn blood spot screening program that will not only use strategies to reduce consanguineous marriages, but also provide early diagnosis and treatment of affected children born from existing marriages. EURORDIS, funded by the EU and brings together rare diseased individuals and patient associations, has recently announced that Italy has the largest newborn blood spot screening program in Europe and called for a similar program to be put on the agenda in other countries (*2*).

3.1. Rare diseases screened in newborns in Turkey from past to present and future plans

Newborn screenings in Turkey started with PKU in 1983 and became a national program in 1994. Subsequently, congenital hypothyroidism was included in the National Screening Program in 2006, biotidinase deficiency in 2008, and cystic fibrosis in 2015, respectively (*30,33*). Finally, the congenital adrenal hyperplasia screening program was started in 2017 (*30*). gradually expanding and becoming national at the beginning of 2022. Approximately 1.2 million babies were born in Turkey in 2020. Currently, there are two National Screening Laboratories. Heel blood is taken within 48-72 hours after birth and results are obtained in 1-2 days on average. It has been stated that the coverage is 97% (*34*).

It is stated in the "Report of the Parliamentary Research Commission Established to Determine the Treatment and Care Methods for ALS, SMA, DMD, MS and Other Diseases with No Definitive Treatment, and the Problems and Solutions of People with These Diseases and Their Relatives", under the Turkish Grand National Assembly, that it is planned to screen an additional 32 diseases which were reported to the commission by the Ministry of Health, General Directorate of Public Health (Table 2) (*35*). If the screening program in Turkey can be expanded with these diseases/disorders, it may develop into one of the most extensive screening programs in the world.

3.2. Opinions of various authorities and institutions/organizations regarding the updated newborn screening criteria

By examining the screening criteria put forward by Wilson and Jungner in 2018, 12 principles under 3 domains were determined and thus updated (*16*). Accordingly, the first domain consists of 3 parts: disease/condition principles *a*) epidemiology of the disease or condition, *b*) natural history of the disease or condition, *c*) target population for screening. The second domain is test/intervention principles, and again consists of 3 parts: *a*) screening test performance characteristics, *b*) interpretation of screening test results, *c*) post-screening test options. The last and third domain, program/system principles, consists of 6 parts: *a*) screening program infrastructure, *b*) screening program coordination and integration, *c*) screening program acceptability and ethics, *d*) screening program benefits and harms, *e*) economic evaluation of screening program, *f*) screening program quality and performance management (*16*).

Additionally, EURORDIS identified 11 key principles for newborn screening in January 2021 (36). These principles can be briefly explained as follows: *i*) screening should be primarily for disease/conditions that can be acted upon, such as treatment, *ii*) neonatal blood spot screening should be embedded within the national system so that it is accessible, *iii*) families of the affected babies diagnosed after the screening should be provided with psychological, *iv*) social and economic support by compatible experts, and all stakeholders should be included in the screening program, *v*) in order to expand the screening program, it should be susceptible to clear, transparent, independent, evidence-based information and policy change and development, *vi*) the management of the screening program should be clear, comprehensive, transparent and accountable, *vii*) the expenses should be considered while determining the diseases to be included or not in the screening program but it should not be conclusive, and should be in line with the most recent evidence, *viii*) all stakeholders should be informed and educated about rare diseases and the screening program, *ix*) the process should be standardized for Europe in terms of quality and uniformity, *x*) blood spot samples should be stored in the national biobank for research purposes, holding appropriate security measures, *xi*) ERN affiliated centers should be integrated into the care and should be considered as preferential partners in providing recommendations on the screening policies.

3.3. Importance, benefit, necessity and limitations of the newborn screening

Newborn blood spot screening has some advantages and disadvantages. These should also be considered when determining the diseases/disorders to be screened (*37,38*). Expanded newborn screening programs have been shown in various studies to be cost-effective, affect the quality of life, and reduce mortality and morbidity (*39*). Lindner *et al*. (*10*) evaluated the expanded newborn blood spot screening in Germany and found that physical and

Table 2. Diseases/Conditions Planned to be Included in the Screening Program of the Ministry of Health, General Directorate of Public Health in Turkey

Disease/Condition Groups	Diseases/Conditions
Fatty Acid Oxidation Disorders	• Medium chain acyl CoA dehydrogenase deficiency • Long chain acyl CoA dehydrogenase deficiency • Short chain acyl CoA dehydrogenase deficiency • Multiple acyl CoA dehydrogenase deficiency (Glutaric acidemia type II) • Long chain hydroxyacyl CoA dehydrogenase deficiency • Trifunctional protein deficiency
Carnitine Cycle Disorders	• Carnitine transporter deficiency • Carnitine palmitoyl transferase I deficiency • Carnitine palmitoyl transferase II deficiency • Carnitine/acyl carnitine translocase deficiency
Organic Acidemias	• Methylmalonic acidemia • B-ketothiolase deficiency • 3-OH-3-methylglutaryl-CoA lyase deficiency • 3-methylcrotonyl-CoA carboxylase deficiency • Isovaleric acidemia • 3-methylglutaconyl-CoA hydratase deficiency • 2-methylbutyryl-CoA dehydrogenase deficiency • Isobutyryl-CoA dehydrogenase deficiency • Propionic acidemia • Glutaric acidemia type I • 3-oxothiolase deficiency • Holocarboxylase deficiency
Urea Cycle Disorders	• Argininosuccinate synthetase deficiency (Citrullineemia) • Argininosuccinate lyase deficiency (Argininosuccinic aciduria) • Arginase deficiency
Amino Acid Disorders	• Tetrahydrobiopterin deficiencies • Maple Syrup Urine Disease (MSUD) • Tyrosinemia • Homocystinuria • Cobalamin disorders • Methylenetetrahydrofolate deficiency

*Congenital Adrenal Hyperplasia**

* The screening program of congenital adrenal hyperplasia has been extended nationally since 2022.

Source: (*35*)

cognitive benefits were similar when phenylketonuria was taken as the gold standard. In addition to screening, positive cases have access to diagnostic tests and, as a result, they also have access to appropriate treatments. In this sense, it is critical to evaluate the diseases/disorders to be screened in terms of long-term outcomes (*40*). According to the long-term evaluation at Boston Children's Hospital, the expanded blood spot screening program had increased the mean IQ score and severe clinical outcomes were significantly reduced, indicating the success of the program (*41*). Early diagnosis of some of these conditions where treatment is available is additionally of great importance (*42*). In addition to the mentioned benefits, it is probable to claim that screening has some negative aspects. Since they are not diagnostic, screening tests have a certain percentage of false positives or false negatives, which should be within the limits of society's acceptance. While false-positive results cause unnecessary additional testing and thus additional costs, redundant intervention, and complications for the patient, false-negative results may cause delays in the diagnosis (*37,38*). Another critical problem with screening is the demand for a significant health workforce and infrastructure (*38*).

3.4. A holistic view of public health in screening for rare diseases in newborns

The approach to rare diseases that are planned to be screened with a holistic approach and public health principles is given in Table 3. Although it is difficult to calculate the burden of rare diseases, it is known that childhood is an important cause of mortality and morbidity. It has been shown that of all inborn errors of metabolism, which are mostly covered by neonatal screening programs, the overall birth prevalence estimate was 50.9 per 100,000 live births by Water *et al*. (*43*). It is stated that the inborn errorrs of metabolism are especially responsible for sudden infant deaths and that most of them are diseases that can be screened and treated (*44,45*). In this sense, the issue cannot be seen apart from Sustainable Development Goals (SDG) Goal 3, Target 3.2 which is explained (*46*) "By 2030, end preventable deaths of newborns and children under 5 years of age, with all countries aiming to reduce neonatal mortality to at least as low as 12 per 1,000 live births and under-5

Table 3. The holistic approach with the principle of public health on screening

Disease Onset and Progression				
Preclinic Phase		Clinic Phase	Postclinic Phase	
Primordial prevention	Primary prevention	Secondary prevention	Tertiary prevention	Quadruple prevention
Evaluation of social determinants of rare diseases that are planned to be screened	Defining the risk factors	Reduction and prevention of delay in diagnosis (early diagnosis)	Social and medical rehabilitation	Avoiding fragmented service delivery
Making the infrastructure ready	Planning the intervention for preventive strategy	Providing appropriate, effective and timely treatment options	Interventions to reduce complications and slowdown the prognosis	Avoiding overdiagnosis
Legal regulations	Intervention	Standardization of the treatment protocol		Avoiding overmedication or medical/surgical intervention
		Integration of the decision support systems		Integration of health information systems

Sustainable Development Goal 3, Target 3.2: By 2030, end preventable deaths of newborns and children under 5 years of age, with all countries aiming to reduce neonatal mortality to at least as low as 12 per 1,000 live births and under-5 mortality to at least as low as 25 per 1,000 live births. From the data to policy; epidemiological assessment and monitoring for all phases and strategies, integrity of society, national/international solidarity, public health ethics, equity in health and social care in the context of universal health coverage, financial planning, management of the health workforce, training of healthcare professionals, and establishing the appropriate infrastructure.

mortality to at least as low as 25 per 1,000 live births". Although screening is a secondary prevention strategy, it may be incomplete unless complemented by the other four prevention strategies (*38,47*). To give an example from social determinants, the existence of isolated groups in a society *e.g.* populations with frequent consanguinity marriages may cause some rare diseases to be seen frequently (*43*). Also, screening of hemoglobinopathies such as hemophilia, thalassemia and fragile X syndrome, as well as CF in risky groups; in addition, Tay-Sachs disease, Canavan disease, Familial dysautonomia for Ashkenazi Jews is recommended by Joint SOGC–CCMG Committee (*48*). Also Tay-Sachs disease carrier screening is recommended by the American College of Obstetricians and Gynecologists (ACOG) for women with Ashkenazi Jewish, French-Canadian or Cajun ancestry (*49*).

4. Limitations

This review cannot be considered as a systematic review. It has some limitations, notably at the level of evidence. The limited number of countries examined and the fact that the countries examined are mostly developed countries may be considered as cause for bias. The small and insufficient number of studies conducted in Turkey has made it difficult to evaluate the specifics of the country and therefore caused the recommendations to become limited.

5. Conclusion

The future of newborn screening programs, as an example of secondary prevention, seems to be an important issue that will always find its place in the scientific community, to conserve and build up its emphasis in terms of public health.

In order to achieve the determined goals of sustainable development without wasting time, equitable access to health services should be ensured by considering ethical principles. When current criteria are taken into consideration and examined, it is obvious that an assessment must be made about expanding the newborn blood spot screening program. In this scheme, the epidemiological information, the infrastructure of the screening program, and the economic evaluation of the screening program should especially be scrutinized about rare diseases/conditions that are candidates for screening in Turkey. To begin with, the establishment of a rare disease registry information system in Turkey would provide epidemiological information and outputs such as incidence rate for screening, benefits from treatments and their effects on survival rate, and thus, a hierarchy from data to information to policy. Second, infrastructure projects must be arranged for the expanded screening program, pilot applications can be implemented if necessary, and an appropriate budget should be allocated.

Thirdly, screening programs should be piloted as means of evaluating economic outcomes. Finally, it is evident that there is a need for more studies on the subject in most investigated countries, especially Turkey and they should review their policies in the context of a holistic approach with the principle of public health on screening.

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Original Article

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Comprehensive bioinformatics analysis of susceptibility genes for developmental dysplasia of the hip

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SUMMARY Developmental dysplasia of the hip (DDH) is a multifactorial disease, which occurs under environmental and genetic influence. The etiopathogenesis of DDH has not been fully explained. As research progresses, many candidate genes have been found to be closely related to the occurrence of DDH. In this study, we comprehensively examined 16 susceptibility genes of DDH using bioinformatics. *COL1A1* encodes the pro-alpha1 chains of type I collagen, which is the major protein component of the bone extracellular matrix (ECM). The genes displaying the most statistically significant co-expression link to *COL1A1* are *ASPN*, *TGFB1*, *DKK1*, *IL-6*, *TENM3* and *GDF5*. DKK1, FRZB and WISP3 are components of the Wnt signaling pathway. CX3CR1 and GDF5 regulate chondrogenesis through the canonical Wnt signaling pathway. ASPN could induce collagen mineralization through binding with collagen and calcium. Integrated bioinformatics analysis indicates that ECM, Wnt signaling pathway and TGF-β signaling pathway are involved in the occurrence of DDH. These provide a basis for further exploring the pathogenesis of DDH.

Keywords developmental dysplasia of the hip, bioinformatics, protein-protein interaction, susceptibility gene, Wnt signaling pathway

1. Introduction

Developmental dysplasia of the hip (DDH), also known as congenital hip dislocation or congenital hip dysplasia, is one of the most frequent skeletal anomalies in newborns (*1*). It is characterized by laxity of the joint capsule caused by mild or incomplete formation of the acetabulum, secondary deformity of the proximal femur and complete luxation (*2*). Although early screening and treatment can help DDH children recover better, there are still many with residual malformations, such as re-dislocation, femoral head necrosis, and residual acetabular dysplasia, which may then develop into adult osteoarthritis, and often requires joint replacement. The whole treatment cycle is long and brings a huge burden to the family. How to fundamentally prevent the occurrence of DDH is an urgent clinical issue to be solved. Hence, it is of great importance to explore the etiology and pathogenesis of DDH.

However, the multifactorial etiology and pathogenesis of DDH have not yet been sufficiently clarified. Many studies have shown that genetic, environmental, and mechanical factors play an important role in the occurrence of DDH (*3*). The theory of the autosomal dominant mode with incomplete penetrance is popular. So the genetic factors occupy an important position in the pathogenesis of DDH (*4*). Genes involved in osteogenesis and chondrogenesis and genes associated with the formation of joint structures and connective tissue contribute to the occurrence of this disorder (*2*).

To date, 16 genes with the highest correlation of DDH in different populations have been reported. These include *ASPN, BMS1, CX3CR1, COL1A1, DKK1, FRZB, GDF5, HOXB9, HOXD9, IL-6, PAPPA2, TBX4, TENM3, TGFB1, UQCC1*, and *WISP3(CCN6)* (*3,4*). Changes in some genes, such as *DKK1, WISP3, HOX, UQCC1, TENM3, CX3CR1, PAPPA2* and *FRZB*, directly lead to abnormal formation of fibrous, bone, and cartilage tissue (*5-13*). Abnormal interactions of IL-6 and TGFB1 also produce the same result (*14*). The *COL1A1* gene encodes

the alpha1 chain of collagen, which is the structural component of cartilage. The promoter variations (rs113647555) in *COL1A1* affect joint laxity (*15*). A positive correlation between *GDF5* polymorphisms and DDH has been demonstrated (*16*). TBX4 and ASPN also act as key regulators that affect the number of fibroblasts in tendons and fascia, resulting in relaxation around the hip joint and increasing the risk of dislocation (*17,18*). BMS1 (rs201298233) indirectly affects bone resorption and mineral density by participating in a large protein-protein interaction (PPI) network (*19*). Bioinformatics was used in this study to examine the relationship of 16 reported DDH susceptibility genes, with the expectation of gaining insight into the possible molecular mechanisms of DDH.

2. Materials and Methods

2.1. Phylogenetic analysis and visualization of gene structures

Sequences of *ASPN, BMS1, CX3CR1, COL1A1, DKK1, FRZB, GDF5, HOXB9, HOXD9, IL-6, PAPPA2, TBX4, TENM3, TGFB1, UQCC1*, and *WISP3(CCN6)* in Fasta format as well as their encoding protein sequences were derived from the NCBI database (*https://www.ncbi.nlm.nih.gov/*). The visualization to truly show the location of these 16 genes on the chromosome was performed by the "gene on genome from Fasta" tool of TBtools software. Multiple alignment of their protein sequences was performed using CLUSTAL 2.0 software. A phylogenetic tree was constructed through Molecular Evolutionary Genetic Analysis (MEGA) software. Motif detection of these 16 protein sequences was performed using the MEME tool (*https://meme-suite.org/meme/index.html*), with the number of motifs equal to 15 and classic mode parameters setting (*20*). The obtained motif mining results and gene structure in Fasta format were visualized in "amazing optional gene viewer" of TBtools software.

2.2. Prediction of coexisting proteins and PPI networks

The STRING (*https://cn.string-db.org/*) and the GeneMANIA (*https://genemania.org/*) online tools were used to analyze the interactions of the 16 proteins coded by DDH susceptibility genes. The STRING website was used to obtain the available protein association networks by using the query of Multiple Proteins by names and organism ("Homo sapiens"). The interaction relationship between these 16 proteins was obtained by setting the following parameters: meaning of network edge was set as evidence, text-mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence were all selected as active interaction sources, with a medium confidence value of 0.4 (*21*). In the GeneMANIA online tool, the types of interactions were revealed by choosing the organism "Homo sapiens", and co-expression, co-localization, physical interactions, shared protein domains and pathway were set.

2.3. Expressive tightness analysis of genes

Correlation expression analysis of DDH susceptibility genes was conducted by MEM-Multi Experiment Matrix (*https://biit.cs.ut.ee/mem/index.cgi*) to obtain the experimental research expression matrix of 16 genes (*22,23*). Genes were entered into the text field, A-AFFY-44 collection was chosen and *COL1A1* was used as the reference gene. Other procedures included setting 0.29 as StDev threshold for query gene, choosing StDev as dataset weight, and using 100 as the number of most variant datasets.

2.4. Enrichment analysis of related genes

To explore interacting proteins for the above 16 different proteins, STRING was used. Experiment-based interacting proteins were acquired by setting the parameters as follows: meaning of network edges was set as evidence, active interaction sources were experiment-based only, high confidence value of 0.150, and no more than 50 interactors in 1st shell. As above, GeneMANIA was conducted to obtain interacting proteins for these 16 target proteins. Meanwhile, "Similar Gene Detection" module of GEPIA2 (*http://gepia2.cancer-pku.cn/#index*) was adopted to gain the top 20 correlated genes for these 16 queries (*24*). Interacted proteins predicted from STRING, GeneMANIA and GEPIA2 were compared by Venn analysis (*http://bioinformatics.psb.ugent.be/webtools/Venn/*).

By combing the above two sets of data, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (*https://david.ncifcrf.gov/*) online tools, then visualized with "Cairo" (*https://cran.r-project.org/web/packages/Cairo/index.html*), "stringr" (*https://cran.r-project.org/web/packages/stringr/index.html*), and "ggplot2" (*https://cran.r-project.org/web/packages/ggplot2/index.html*) R packages. Gene Ontology (GO) enrichment of biological process (BP), cellular component (CC), and molecular function (MF) were visualized by "clusterProfiler" R package (*http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html*). $P < 0.01$ was set as the statistical significance threshold value.

2.5. Genetic alteration analysis

For the analysis of alteration in *ASPN, BMS1, CX3CR1, COL1A1, DKK1, FRZB, GDF5, HOXB9, HOXD9, IL-6, PAPPA2, TBX4, TENM3, TGFB1, UQCC1*, and *WISP3(CCN6)*, the cBioPortal (*https://www.cbioportal.*

org/) browser was selected in "TCGA Pan Cancer Atlas Studies" module. The frequency and characteristics of three different types of alteration including mutated gene, amplification and copy number alteration (CNA) were analyzed in all tumors recorded by TCGA databases (*25,26*). The corresponding mutation sites of *PAPPA2* and *TENM3* were conducted through "mutations" module.

3. Results

3.1. Phylogenetic analysis and visualization of gene structures

The locations of DDH susceptibility genes are scattered and spread over 11 chromosomes. There are no collinear genes. *HOXD9* and *FRZB* are located on chromosome 2. *BMS1* and *DKK1* are located on chromosome 10. *HOXB9, COL1A1* and *TBX4* are located on chromosome 17. *GDF5* and *UQCC1* are located on chromosome 20. The other seven genes are located on chromosomes 1, 3, 4, 6, 7, 9, and 19, respectively. It is worth noting that *GDF5* and *UQCC1* are relatively close (Figure 1A). A previous study has shown that abnormal bone growth and development in humans is associated with common variants in the *GDF5-UQCC* region (27).

The motif structures of 16 proteins are quite different, which reflects the complexity of DDH at the protein macromolecule level. Pathogenically, HOXB9 and HOXD9, which belong to the same family, are structurally similar, which is also consistent with the gene structure results (Figure 1B). In addition to the gene structures and phylogenetic tree, we also compared the positions and numbers of exons and introns of 16 genes (Figure 1C). The results showed that there is a diversity of structures for DDH susceptibility genes, among which *TENM3* is the largest, *PAPPA2* is second, *DKK1* is the smallest, and there is no good evolutionary relationship among these genes.

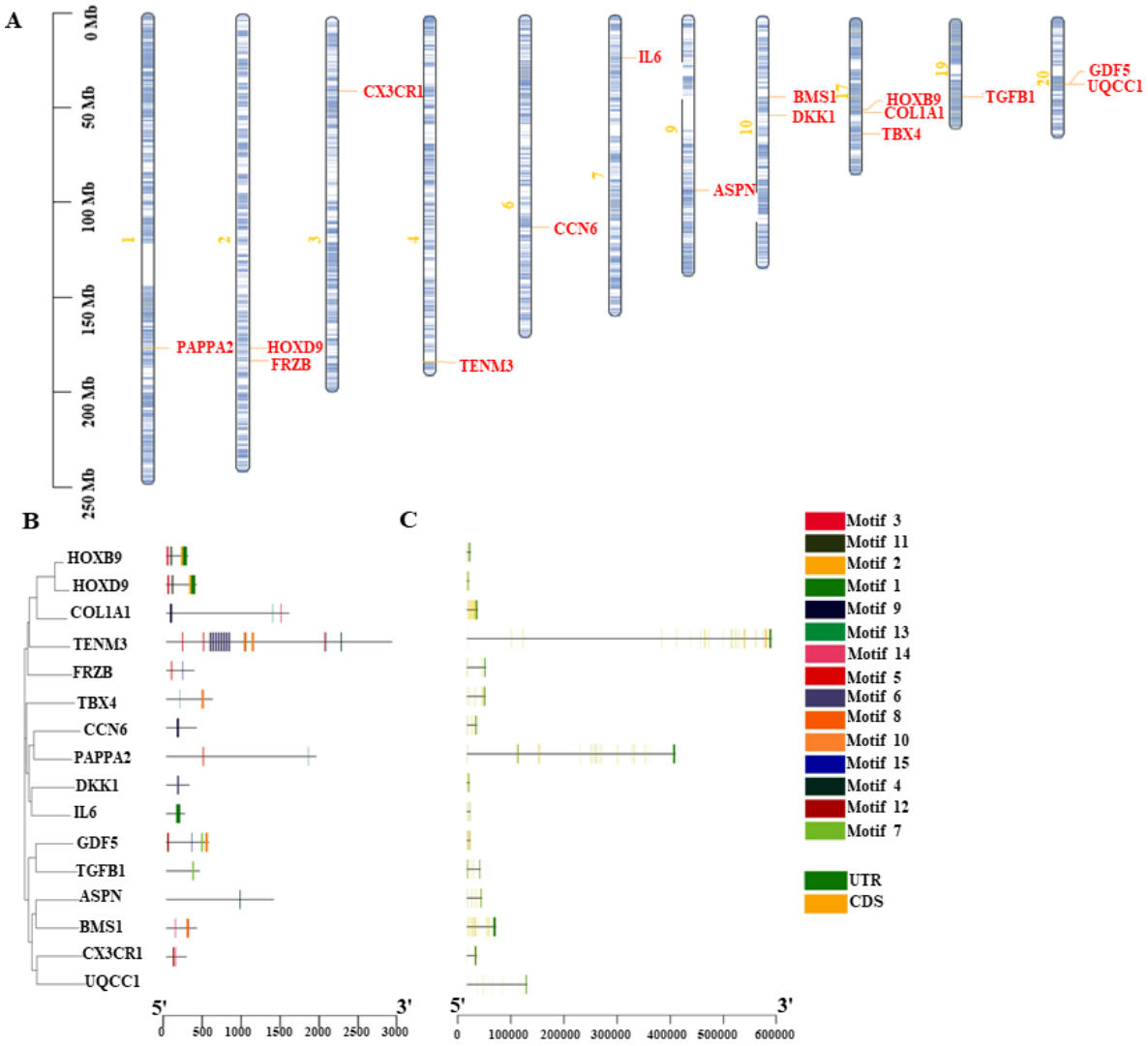

Figure 1. Phylogenetic analysis and visualization of chromosomal location and structures of 16 DDH susceptibility genes. (A) Chromosome location; **(B)** Phylogenetic analysis; **(C)** Gene structure.

3.2. Prediction of coexisting proteins and PPI networks

PPI analysis conducted by STRING indicated that co-expression is the most common among all interactions of 16 analyzed proteins, and it is worth noting that *COL1A1* encoding protein has a co-expression relationship with four proteins, namely ASPN, GDF5, DKK1, and IL6 (Supplementary Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=98*, Figure 2A). The highest score between *COL1A1* encoding protein and ASPN protein was 0.38. The prediction results of the GeneMANIA database showed that these 16 proteins were associated with TSR1, DKK2, DKK3, RSPO1, TENM2, DKK4, GTF3A, ITGA11, TENM1, KREMEN2, TENM4, IL17A, MED12, HOXC9, HOXA9, PAPPA, COL1A2, FZD8, CSF3 and VEGFD, a total of 20 proteins (Table 1, Figure 2B). It is worth noting that TGFB1 and GDF5 share common domains. TGFB1 and GDF5 are members of the TGF-β superfamily, and both act as important regulators in bone and cartilage formation in DDH-related pathways (*3*).

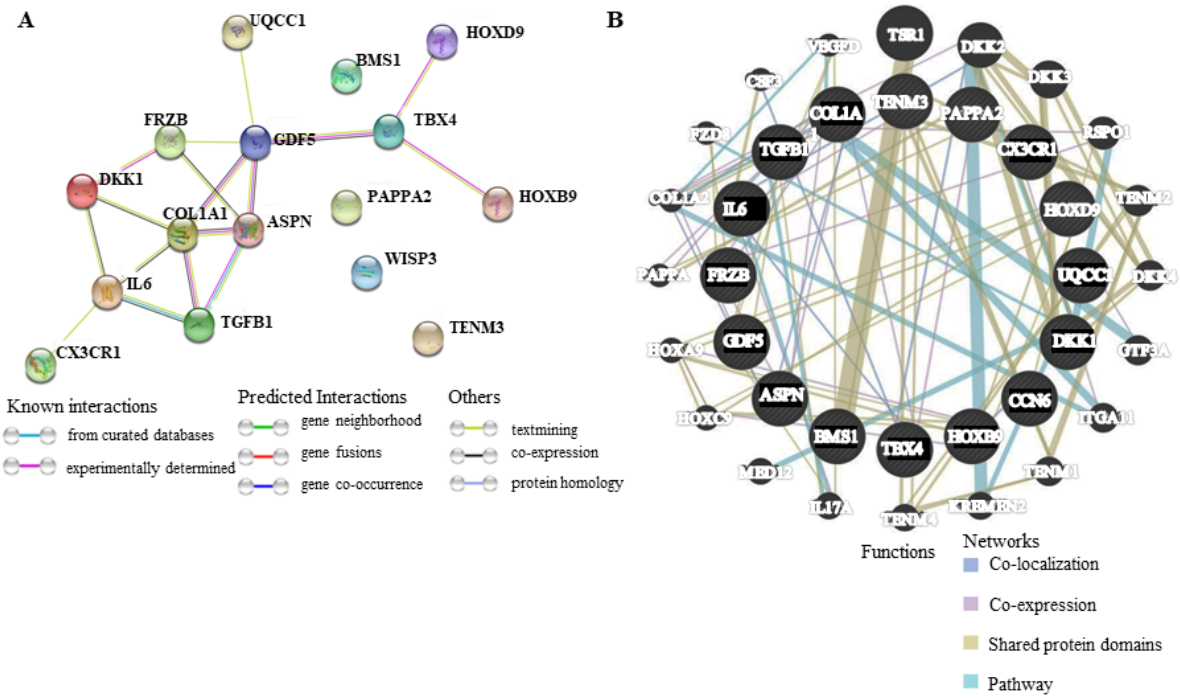

Figure 2. Predicted protein-protein interaction. (A) The interaction networks between 16 DDH susceptibility genes; **(B)** Protein interaction networks between susceptibility genes and 20 related genes.

Table 1. Top 20 encoding genes of interacted proteins indicated by GeneMANIA

Gene	Description	HGNC	Rank
TSR1	TSR1 ribosome maturation factor	25542	1
DKK2	dickkopf WNT signaling pathway inhibitor 2	2892	2
DKK3	dickkopf WNT signaling pathway inhibitor 3	2893	3
RSPO1	R-spondin 1	21679	4
TENM2	teneurin transmembrane protein 2	29943	5
DKK4	dickkopf WNT signaling pathway inhibitor 4	2894	6
GTF3A	general transcription factor IIIA	4662	7
ITGA11	integrin subunit alpha 11	6136	8
TENM1	teneurin transmembrane protein 1	8117	9
KREMEN2	kringle containing transmembrane protein 2	18797	10
TENM4	teneurin transmembrane protein 4	29945	11
IL17A	interleukin 17A	5981	12
MED12	mediator complex subunit 12	11957	13
HOXC9	homeobox C9	5130	14
HOXA9	homeobox A9	5109	15
PAPPA	pappalysin 1	8602	16
COL1A2	collagen type I alpha 2 chain	2198	17
FZD8	frizzled class receptor 8	4046	18
CSF3	colony stimulating factor 3	2438	19
VEGFD	vascular endothelial growth factor D	3708	20

3.3. Expressive tightness analysis of genes

The correlation matrix for expression data of the 16 genes was obtained from the MEM-Multi Experiment Matrix open database. We used *COL1A1* as the reference standard. The results showed that there were higher expression densities between *COL1A1* and 6 genes: *ASPN*_(219087_at), *TGFB1*_(203085_s_at), *DKK1*_(204602_at), *IL-6*_(205207_at), *TENM3*_(219523_s_at) and *GDF5*_(206614_at). The scores were 1.3E-34, 1.49E-25, 2.57E-24, 6.67E-22, 1.31E-17 and 7.61E-12, respectively (Figure 3). It indicates that *ASPN, TGFB1, DKK1, IL-6, TENM3, GDF5* and *COL1A1* were more closely expressed in the corresponding experimental projects. Although the expression of other genes was correlated, the expression affinity was not significant.

3.4. Enrichment analysis of related genes

To ensure the reliable protein-protein interaction predication, experiment-based interacting proteins for the 16 DDH related proteins were analyzed by STRING and GeneMANIA (Figure 4A, Supplementary table S2, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=99*). Correlated proteins for the 16 proteins were predicted by GEPIA2 (Supplementary table S2, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=99*). Venn analysis demonstrated that three proteins, including CSF3, RSPO1 and COL1A2, were predicted by both GEPIA2 and GeneMANIA. LTBP1 and IL6ST were identified from the intersection analysis of STRING and GEPIA2 (Figure 4B).

KEGG pathway enrichment analysis suggested that the analyzed DDH susceptibility genes were mainly enriched in Wnt, TNF and TGF-β signaling pathways, signaling pathways regulating pluripotency of stem cells, ribosome biogenesis, regulation of actin cytoskeleton, focal adhesion, ECM-receptor interaction, and so on. Most notably, genes enriched in ribosome biogenesis in eukaryotes were greater than 20 and -log10 (*p*-value) greater than 12 (Figure 4C).

GO analysis demonstrated the enriched biological process, which included ncRNA processing, ribonucleoprotein complex biogenesis, ribosome biogenesis, rRNA metabolic process, rRNA processing, and so on (Figure 4D). Cellular components were enriched in 90s preribosome, collagen-containing extracellular matrix, collagen trimer, preribosome, small-subunit processome (Figure 4E). Molecular functions were mainly enriched in extracellular matrix structural constituent, glycosaminoglycan binding, growth factor binding, snoRNA binding, and transforming growth factor β-activated receptor activity (Figure 4F). Notably, the main function of BMS1 is related to eukaryotic ribosome biosynthesis. At present, there are few studies on the correlation between BMS1 and DDH. One study has shown that variants of the *BMS1* gene are associated with alterations in bone resorption and mineral density (*19*).

3.5. Genetic alteration for genes

Prevalence and characteristics of genetic alteration of *ASPN, BMS1, CX3CR1, COL1A1, DKK1, FRZB, GDF5, HOXB9, HOXD9, IL-6, PAPPA2, TBX4, TENM3, TGFB1, UQCC1*, and *WISP3(CCN6)* in 33 types of cancer in TCGA database were acquired. A total of 10,967 samples originating from 10,953 patients were tested for five different types of genetic alteration, including mutations, fusions, amplifications, deep deletions, and multiple alteration. Mutation was the predominant type in most tumors as indicated (Figure 5A). After observing the mutations of every gene, it was found that the highest mutation of 25.9% for *PAPPA2* was identified in melanoma (Figure 5B and 5C). The mutation frequency of *TENM3* in melanoma was as high as 24.8% (Figure 5D and 5E). In addition, the KEGG enrichment results also revealed that the related genes of these 16 genes are highly involved in cancer pathways. We found that the change of arginine to leucine or histidine at position 324 of FRZB was identified in esophageal adenocarcinoma, endometrioid carcinoma and lung adenocarcinoma. A variant of *FRZB* (rs7775), with a cysteine replacement at position 324, was reported in DDH (*13*). Polymorphism at the same locus leads to the different clinical symptoms of the disease. Similarly, glutamine to lysine change at position 56 was identified in skin melanoma. Polymorphism of *CCN6* (rs1230345), resulting in a glutamine to histidine change, was associated with DDH development (*6*). Notably, fusion mutations of *UQCC1* and *GDF5* lead to the development of lung squamous cell carcinoma. Mutations in *GDF5* affect transcriptional processes that ultimately affect joint angles to exacerbate DDH progression (*27*).

4. Discussion

Mild acetabular dysplasia or severe hip dislocation during infancy and early childhood development is defined as DDH. DDH could cause notable pain and osteoarthritis by early adulthood (*28*). It is associated with a variety of risk factors, such as female gender, intrauterine breech, and positive family history (*29*). Postural is one of the risk factors. About 2% to 3% of normal newborns are breech births, but breech birth rate in children with DDH is as high as 16% (*30*). One in 35 breech-birth girls are DDH patients (*31*). DDH is more likely to occur in newborns wrapped in knee and hip extension position. On the contrary, if hip abduction flexion is kept, the incidence is lower (*32*). In DDH rabbit model, the thickening of acetabular cartilage in young rabbits and fibrosis in adult rabbits were found. The expression of integrin β, type I collagen and type II collagen were changed in the process of cartilage thickening and

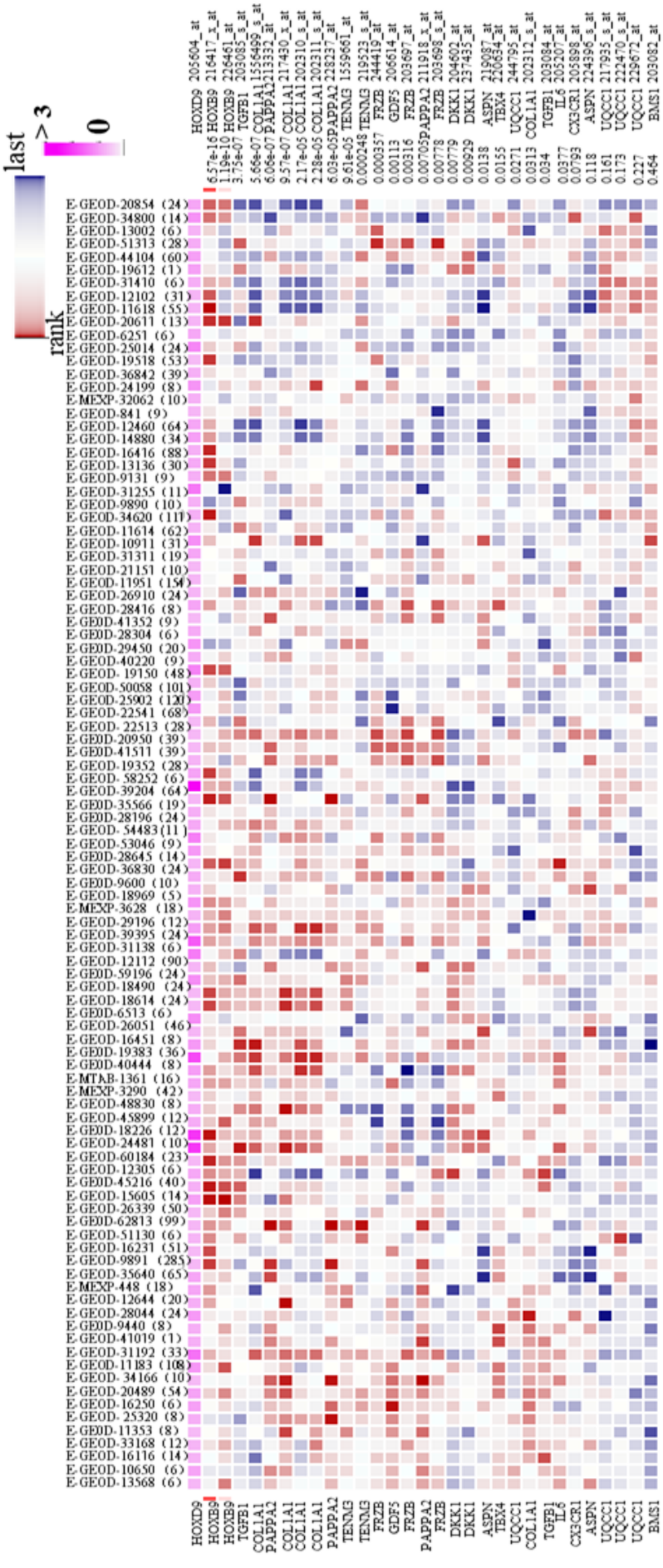

Figure 3. Co-expressed 16 susceptibility genes predicted by MEM. *COL1A1* was set as the reference gene.

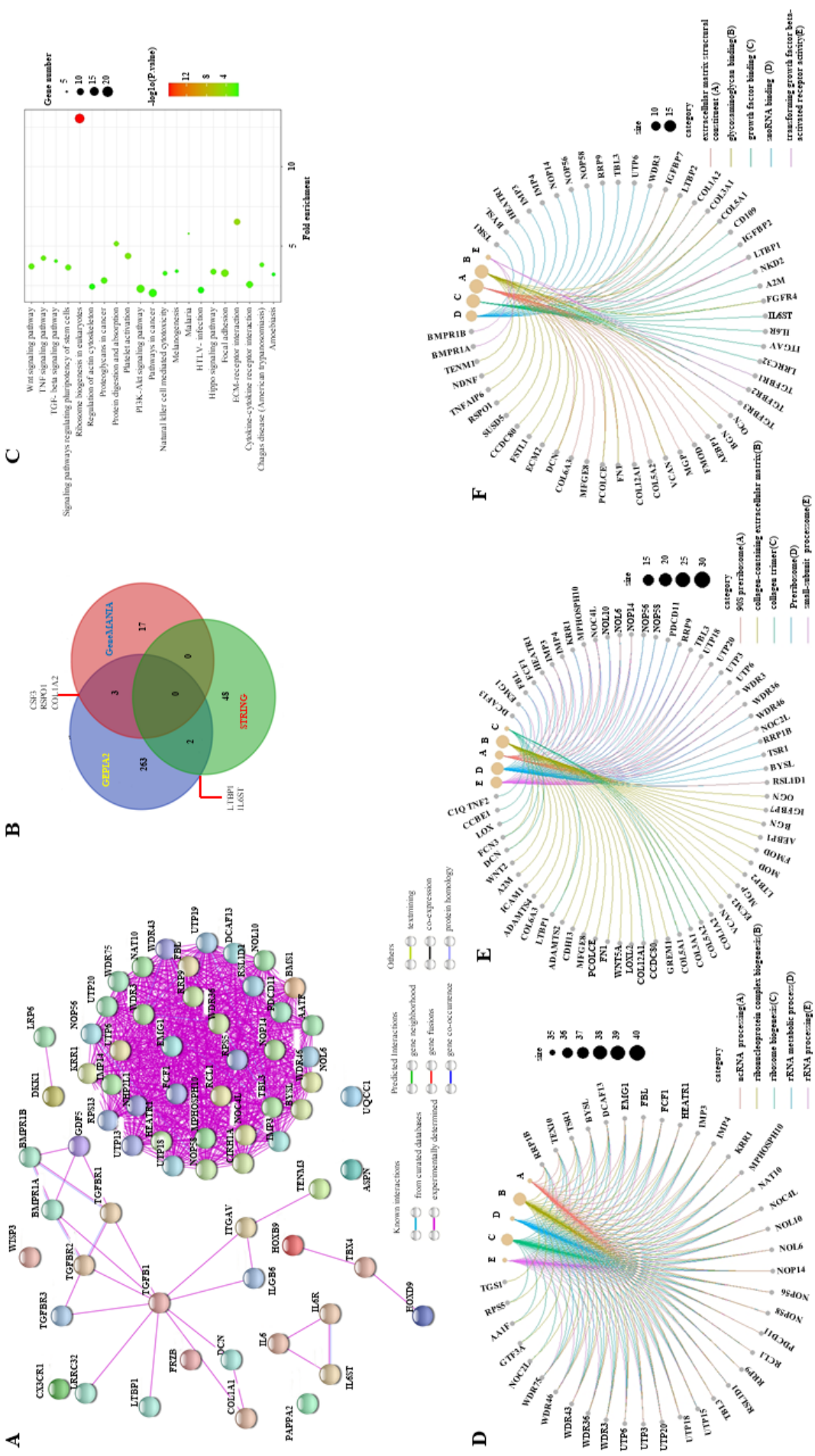

Figure 4. Protein-protein interaction and enrichment analysis. (A) PPI network as analyzed by STRING; **(B)** Venn diagrams showing overlap of the number of proteins predicted by STRING, GeneMANIA, and GEPIA2; **(C)** KEGG pathway enrichment analysis for interacted proteins; **(D-F)** Enrichment analysis of biological process (BP), cellular component (CC) and molecular function (MF).

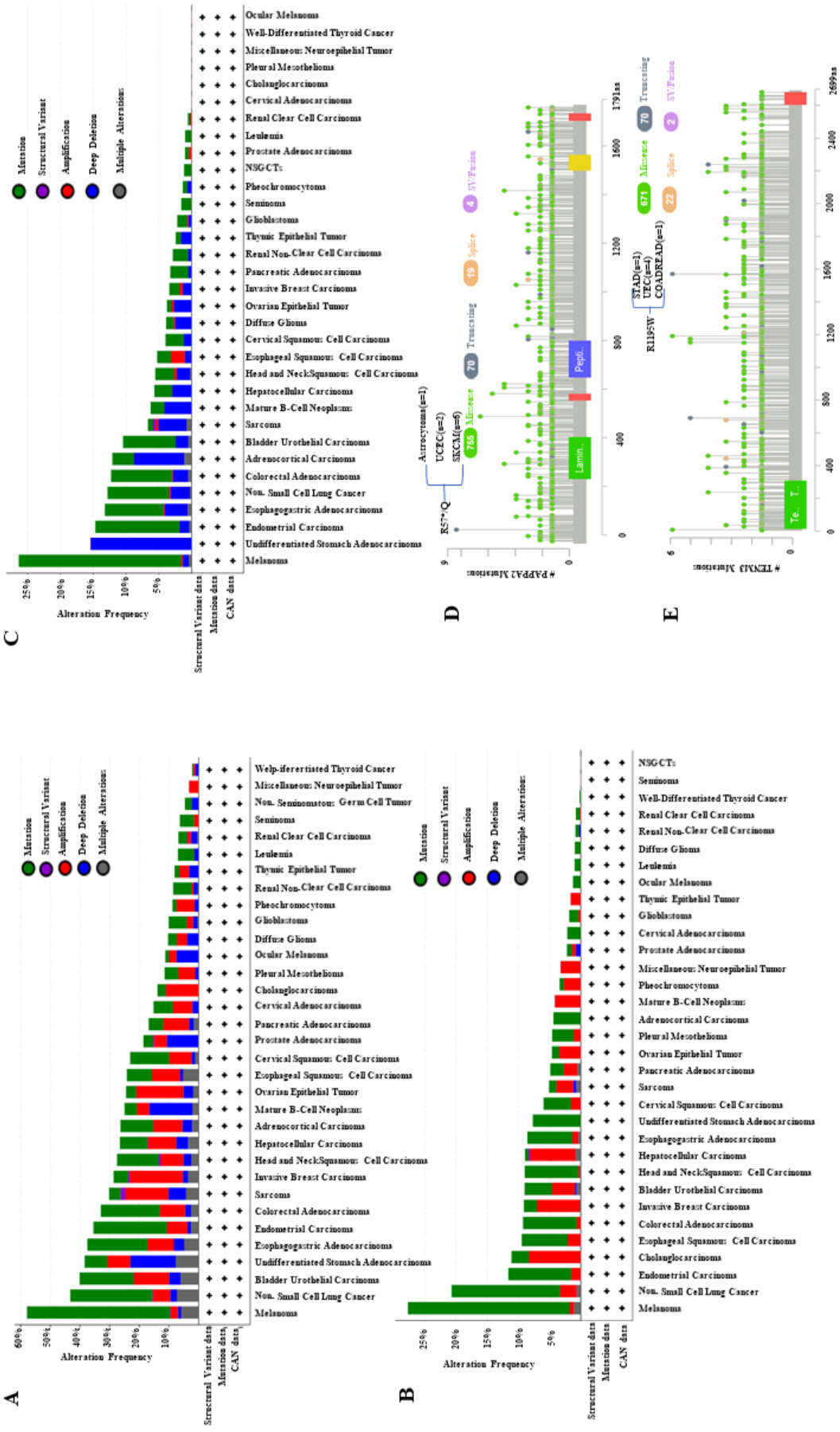

Figure 5. Genetic alteration for 16 DDH susceptibility genes in different tumors of TCGA using the cBioPortal tool. (A) Mutation types; **(B-C)** The mutation features and mutation site of PAPPA2; **(D-E)** The mutation features and mutation site of *TENM3*.

fibrosis, suggesting that mechanical conduction signal pathway is involved in the degeneration of acetabular cartilage (*33*). Meanwhile, both the ratio of different types of collagen and the size of collagen fibrils changed, possibly due to the abnormal collagen metabolism (*34*). Collagen is one of the main components of extracellular matrix and it provides stability to the matrix. Variation in the *COL1A1* gene promoter is associated with DDH in Chinese Han (*15*). *ASPN* encodes a cartilage extracellular protein that belongs to the small leucine-rich proteoglycan family (SLRP). It binds collagen and calcium and induces collagen mineralization (*35*). In *ASPN*-/- mice, biomechanical phenotype was changed, along with relatively thinner collagen fibrils, higher expression of collagen genes, increased chondroitin/dermatan and versican proteoglycans, and increased amount of decorin and biglycan protein (*36*). Following the comprehensive bioinformatics analysis, we proposed that the interaction of collagen and ASPN contributes to the mechanical change and plays a role in DDH cartilage degeneration. Enriched KEGG and GO analysis indicated that DDH susceptibility genes are involved with ECM pathway, collagen-containing ECM and glycosaminoglycan binding. Other proteins, like integrin, were identified, from PPI analysis, to interact with DDH susceptibility genes. Integrins participate in cell-cell and cell-matrix interactions. Integrin-ECM was reported in osteogenesis and the inhibition of chondrogenesis (*37*).

Wnt signaling pathway is one of the main pathways enriched in DDH. FRZB is a secreted protein, functioning as a modulator of Wnt signaling through direct interaction with Wnts. FRZB was reported to regulate chondrocyte maturation and long bone development. Its expression in DDH joint tissue was significantly higher than that in the control group (*13*). FRZB mediated the cell adhesion pathway and cell spreading by regulating integrin expression (*37*). Polymorphisms rs2242070 and rs3768842 of *FRZB* were involved in DDH (*37*). DKK1 binds to the LRP6 co-receptor and inhibits canonical beta-catenin-dependent Wnt signaling pathway, which is critical for chondrogenesis and joint formation (*38*). WISP3 is a member of the WNT1 inducible signaling pathway (WISP) protein subfamily, which belongs to the connective tissue growth factor (CTGF) family. It is the pathogenic gene for progressive pseudorheumatoid dysplasia, a joint disease characterized by degeneration of the cartilage between bones (*1*). Meanwhile, CX3CR1 regulates chondrocyte proliferation and apoptosis through the Wnt signaling pathway and this is associated with the inflammatory reaction of osteoarthritis (*39*). GDF5 is a ligand of the TGF-β superfamily, which could induce chondrogenesis in rat limb bud cells (*40*). GDF5 regulates MMP13 expression *via* DKK1 mediated Wnt/β-catenin signaling pathway in chondrocytes (*41*). RSPO1, as one of the interaction proteins predicted, can affect the differentiation process of osteoblasts and chondrocytes by stimulating the Wnt signaling pathway, maintaining articular cartilage homeostasis and joint formation (*42,43*). Similar to DKK1, it has an important role in tissue repair and fibrosis (*44*). Additionally, it was reported to activate TGF-β signaling and suppress the tumorigenesis of colon cancer (*45*).

TGFB1 and IL-6 are pro-inflammatory cytokines, which take part in the pathogenesis of hip osteoarthritis (*46*). They are involved in the bone remodeling process (*47*). The *HOX* genes encode a conserved family of transcript factors that control morphogenesis and embryonic skeletal formation through endochondral ossification (*48*). A former study has shown that some *HOX* genes encode transcription factors that are important to skeletal development and play a role in embryonic limb development (*49*). Their specific role in the DDH is still unknown. In osteoarthritis, HOTAIR, an lncRNA HOX transcript antisense RNA, could enhance the expression of SGTB by acing as miR-1277-5p sponge, and hence regulates LPS-induced chondrocyte apoptosis and inflammation (*50*).

Through comprehensive bioinformatic analysis, we identified the interactions among susceptibility genes and signaling pathways correlated with DDH. The results in this study can eventually provide novel clues for understanding the molecular mechanisms underlying the pathogenesis of DDH.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Communication

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Intravesical $MgSO_4$ for the treatment of BCG refractory T1 G3 bladder cancer: Preliminary results on efficacy and safety

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SUMMARY An urgent need of therapy exists for patients with high-risk non-muscle invasive bladder cancer (NMIBC) for whom Bacillus Calmette-Guérin (BCG) refractory treatment has failed. We investigated the role of intravesical magnesium sulfate ($MgSO_4$) therapy in the management of BCG refractory T1 high grade (G3) NMIBC. Between January 2018 and July 2021, we performed a prospective trial enrolling participants with T1 G3 NMIBC refractory in BCG therapy. All patients included were considered ineligible for or have refused to undergo radical cystectomy. Subjects are enrolled into a single treatment group of a fixed dose of intravesical $MgSO_4$. The intravesical solution was given for 3 h bi-weekly × 6 then once per week for 12 months. Cystoscopic surveillance was performed every 3 months. Endoscopic resection was performed if suspicious findings were identified on surveillance cystoscopy to establish pathologic diagnosis. Oncological outcomes and any side effects were reported during follow-up. A total of 8 patients who received intravesical $MgSO_4$ for refractory TG3 tumors were included in our study. The median follow-up time was 29 months (range from 23 to 36). 62.5% of the patients (5/8) achieved a complete response to intravesical $MgSO_4$, while 25% of the patients (2/8) had a partial response and 12.5% (1/8) had persistent disease. None of the patients had disease progression. None of the patients experienced hypermagnesemia. In patients with pTG3 tumors who were refractory to BCG therapy, intravesical $MgSO_4$ was a well-tolerated and potentially effective regimen.

Keywords magnesium sulfate, intravesical, bladder cancer, non-muscle invasive

1. Introduction

Non-muscle invasive bladder cancer (NMIBC) is a challenging disease, with a high risk of recurrence and even progression to muscle invasive disease (*1*). Bacillus Calmette-Guérin (BCG) is the only intravesical agent shown to reduce the risk of progression of NMIBC to muscle-invasive disease but it still fails in up to 40% of patients (*2*). Currently, the best option for these patients is radical cystectomy. Novel treatment modalities for BCG failure include intravesical chemotherapy, BCG re-challenge or combination of BCG with IFN-α2β, valrubicin, radiotherapy, electromotive drug administration, vicinium, chemohyperthermia, photodynamic therapy, gene therapy, vaccine therapy, and immunotherapy (*3*). An urgent need for therapy exists for patients with high-risk NMIBC for whom BCG has failed and who seek further bladder-sparing approaches.

2. The rationality to use magnesium sulfate to induce toxicity of cancer cells

In animal models magnesium sulfate ($MgSO_4$) can induce cytotoxicity of cancer cells and release pro-inflammatory cytokines (*4*). The objective of this pilot study was to test if intravesical $MgSO_4$ therapy can manage BCG refractory T1 high grade (G3) NMIBC in patients for whom cystectomy was not an option due to medical reasons, or was offered but refused.

3. Trial design

Between January 2018 and July 2021, we performed a prospective trial enrolling participants with T1 G3 NMIBC refractory in BCG therapy and who are considered ineligible for or have refused to undergo radical cystectomy. BCG refractory disease was defined as biopsy-proven recurrence T1 G3 tumor at 3 months of

receiving a 6-week induction course of BCG, or if a high-grade tumor is present after 3 months and/or at 6 months after either re-induction or first course of maintenance of BCG therapy, or if a high-grade tumor appears during BCG maintenance therapy. All patients with initial or subsequent pathology that revealed carcinoma in situ (CIS) are excluded. Fully resected disease at study entry is also required. Re-resection was performed for all patients. All patients included in the study had the same initial pathology on re-resection specimen. Patients who had any upgrading in the re-resection pathology were excluded. Information regarding patient demographics, time to recurrence, response to $MgSO_4$ therapy, and side effect profile was recorded.

Subjects are enrolled into a single treatment group of a fixed dose of intravesical $MgSO_4$. The intravesical solution was given for 3 h bi-weekly × 6 then once per week for 12 months. Magnesium sulfate heptahydrate 50% (2 mmol Mg^{2+} in 1 ml) was used. Two 10 ml ampoules containing 20 mmol Mg^{2+} were diluted in 30 cc of normal saline solution. Solution preparation was supervised by a clinical pharmacist. The reconstituted $MgSO_4$ solution is injected into the bladder by gravity flow *via* a Foley catheter. Before each injection, a urinalysis was performed to exclude urinary infection. Patients should empty their bladder before each $MgSO_4$ administration. The patient should be repositioned from left side to right side and also should lie upon the back and the abdomen, changing these positions every 15 minutes to maximize bladder surface exposure to the agent. It is important to note that at least 8 weeks should be the interval between intravesical therapy and last bladder resection.

Any side effects were reported. Even though systemic side effects were not expected, all patients using bladder instillation of $MgSO_4$ were tested for magnesium level three times (before, 4 hrs, and 12 h after therapy). Serum magnesium concentration > 2.6 mg/dL indicates hypermagnesemia. Urological follow-up was done by the same urologist who was blinded to the treatment solution.

Cystoscopic surveillance was performed every 3 months. Endoscopic resection was performed if suspicious findings were identified on surveillance cystoscopy to establish pathologic diagnosis. All specimens were examined by a single, experienced pathologist blinded to the treatment protocol. Complete response is considered if no tumor was identified during follow-up; partial response if lower grade and/or stage tumor was identified compared to before $MgSO_4$ therapy; persistent disease if the same grade and stage tumor was identified; or disease progression if higher grade and/or stage tumor was identified.

Adverse events observed after the administration of $MgSO_4$ for the follow-up period were recorded at regular visits by the same urologist who was blinded to the study protocol and intervention.

Table 1. Patient baseline characteristics

Patient number	Age (years)	Gender	Time since last BCG instillation
1	56	Male	6 months
2	62	Male	9 months
3	63	Male	10 months
4	64	Female	5 months
5	71	Female	4 months
6	66	Male	3 months
7	69	Female	3 months
8	59	Female	2 months

BCG: Bacillus Calmette-Guérin.

The study was done at Al Zahraa Hospital in Beirut. It was approved by its IRB (approval No. 2018.2). Informed signed consent was obtained from all patients and confirmed by the IRB. The authors confirm the availability of, and access to, all original data reported in this study.

4. Main findings

A total of 8 patients who received intravesical $MgSO_4$ for refractory TG3 tumors were included in our study. Baseline characteristics are summarized in Table 1. The median age of the patient was 66 years, the male: female ratio was 1. The median time since last BCG instillation was 4.5 months.

The median follow-up time was 29 months (range 23 to 36). 62.5% of the patients (5/8) achieved a complete response to intravesical $MgSO_4$, while 12.5% of the patients (1/8) had persistent disease. 25 % of the patients (2/8) had a partial response while on therapy. None of the patients experienced disease progression. The time since the last BCG instillation for patients who had persistent disease was 3 months (patient 6), whereas the time since the last BCG instillation for patients who had a partial response (patient 7 and 8) was 3 and 2 months respectively. Oncologic outcomes after intravesical $MgSO_4$ therapy are summarized in Table 2.

There were no serious adverse events reported in the treatment group. None of the patients experienced hypermagnesemia and the serum magnesium level did not change after therapy in all patients.

5. The effect of $MgSO_4$ on cancer cells

$MgSO_4$ can induce cytotoxicity of cancer cells. Zhang *et al*. conducted a study to assess the possible cytotoxicity of $MgSO_4$ on human gastric adenocarcinoma cells (AGS) and gastric mucosa in mice. $MgSO_4$ treatment decreased the viability of AGS cells in a concentration-dependent manner and showed a significant decrease in viability. $MgSO_4$ influences cytokine secretion. In AGS cells, the secretion of IL-1β and IL-8 decreased, and that of TNF-α increased with increasing concentrations

Table 2. Oncologic outcomes after intravesical $MgSO_4$ therapy

Patient number	Complete response	Partial response	Persistent disease	Disease progression	Follow-up time (months)	Pathology identified in patients who had a partial response to therapy
1	Yes	No	No	No	23	N/A
2	Yes	No	No	No	29	N/A
3	Yes	No	No	No	25	N/A
4	Yes	No	No	No	26	N/A
5	Yes	No	No	No	30	N/A
6	No	No	Yes	No	36	N/A
7	No	Yes	No	No	32	pTaHG
8	No	Yes	No	No	31	pTaHG

N/A: not applicable; HG: high grade; $MgSO_4$: Magnesium sulfate.

of $MgSO_4$ (*4*). In another study, $MgSO_4$ caused oxidative stress by generating reactive oxygen species (ROS) which caused DNA damage in testicular cells (*5*). $MgSO_4$ could also affect apoptosis of cancer cells by inducing a change in expression of Fas ligand (FasL) and Fas receptor (FasR) (*6*).

6. Important oncological outcomes in this trial

In our trial, none of the patients had disease progression, while 1 patient had persistent disease and 2 others had a partial response to therapy. Down-staging was observed in patients who had a partial response. We noted that the last time of BCG exposure for patients who had a persistent or partial response to $MgSO_4$ was shorter than that of other patients included in the trial (2 and 3 months).

Previously published results indicated that $MgSO_4$ reduced cancer cell viability. In this paper, we used $MgSO_4$ to study its effect on bladder tumor cells. Oncological outcomes and results of recurrence and progression, indicate that $MgSO_4$ could affect the viability of tumor cells within the bladder. Therefore, further research will be required to determine how $MgSO_4$ could induce apoptosis of urothelial tumor cells and to investigate the molecular mechanisms involved.

In conclusion, in patients with pTG3 tumors who were refractory to BCG therapy, intravesical $MgSO_4$ was a well-tolerated and potentially effective regimen.

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Letter

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Diagnosing Alström syndrome in a patient followed up with syndromic obesity for years

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SUMMARY Alström syndrome (AS) is a rare autosomal recessive monogenic disorder caused by mutations of the *Alström syndrome 1* (*ALMS1*) gene, located on chromosome 2p13. It is a progressive multisystemic disease characterized mostly by obesity, sensorineural hearing loss, visual impairments, cardiomyopathy, insulin resistance and/or type 2 diabetes mellitus (T2DM), metabolic dysfunctions, non-alcoholic fatty liver disease, and chronic progressive kidney disease. Generally, the first clinical symptoms of the disease appear in the first years of life with a major variation of onset age. In this study, we aimed to examine the molecular diagnosis of a 6-year-old patient with suspected AS clinical symptoms. After applying clinical exome sequencing (CES) in the patient we found a homozygous deletion in exon 8 at the *ALMS1* gene (c.2311_2312del). We identified a homozygous frameshift mutation. The reported variant was pathogenic according to the criteria of the American College of Medical Genetics and Genomics (ACMG). Thus, the patient was diagnosed with AS as a result of the combined clinical phenotype and genetic tests results. We hope the variant we found can expand the spectrum of *ALMS1* variants in AS.

Keywords *ALMS1* gene, biallelic mutations, obesity, rare diseases

Alström syndrome (AS) is a rare genetic disorder caused by biallelic mutations of the *Alström syndrome 1* (*ALMS1*) gene located on chromosome 2p13 (*1*). *ALMS1* gene was first identified in 2002, it has a 220 kb size and contains 23 exons (*2*). It encodes a 41,1-kDa protein which is made up of 4,169 amino acids (*2*).

AS is an ultra-rare disease with a prevalence of one in 1,000,000. It is mostly characterized by ophthalmological abnormalities (photophobia, nystagmus, cone-rod dystrophy, loss of light perception) sensorineural hearing loss, obesity, cardiomyopathy, insulin resistance, metabolic dysfunctions, non-alcoholic fatty liver disease, and progressive kidney disease (*2*). Different ages of onset of the disease and high variability of clinical symptoms reduce the possibility of making the diagnosis in the early stages. That's why genetic analysis plays an important role in early diagnosis.

A 6-year-old female patient was consulted to our department from the pediatric endocrinology clinic due to dysmorphic features and obesity. She was born on time to healthy Turkish parents who were 5th-degree relatives. No extraordinary situation was observed in the prenatal follow-up and delivery of the patient.

The weight and height of the patient were 44,4 kg (>P97, 4.20 SDS), 131,5 cm (P97<, 2,77 SDS) respectively. Her physical examination revealed truncal obesity, wide forehead, high arched palate, acanthosis nigricans on the neck, and buffalo hump, tapered finger, and simian crease on the right hand (Online Figure, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=92*). At 6 months of age bilateral nystagmus and photophobia were observed in her eye evaluation. Later, ophthalmoscopy showed diffuse dystrophy of the retinal pigmented epithelium at 5 years old. In the follow-up of the patient from the age of 3, a considerable weight gain was observed in anthropometric measurements according to age and height of the patient (Table 1). In the pubertal evaluation of the 5-year-old patient, thelarche was found consistent with Tanner stage 3 without axillary and pubic hair growth. Later in the laboratory tests of the patient, fasting glucose: 87 mg/dL, HbA1c: 5.4% (normal range: 4.8-5.9), insulin: 61.69 (normal: 2.6-24.9) µIU/mL, FSH: 0.92 mIU/mL (22.02.2019), LH < 0.3 mIU/mL, Estradiol: 13 pg/mL (6-27) were detected. The skeletal survey result was compatible with 8 years of age. Due

Table 1. Patient's height and weight follow-up over the years

Date of Examination	Age (years)	Weight (kg)	SDa	Height (cm)	SDa	BMI (kg/m^2)	SDa
2017	3	29.0	+4.77	101.0	+0.47	28.4	+5.04
2018	4	34.1	+5.12	111.3	+1.58	27.5	+4.61
2019	5	38.6	+4.78	118.0	+1.94	27.7	+3.96
2020	6	42.7	+3.99	129.4	+2.33	25.5	+3.07
2021	7	44.8	+3.65	132.8	+2.36	25.4	+2.80

SDa: Standard Deviation Analysis, BMI: Body Mass Index

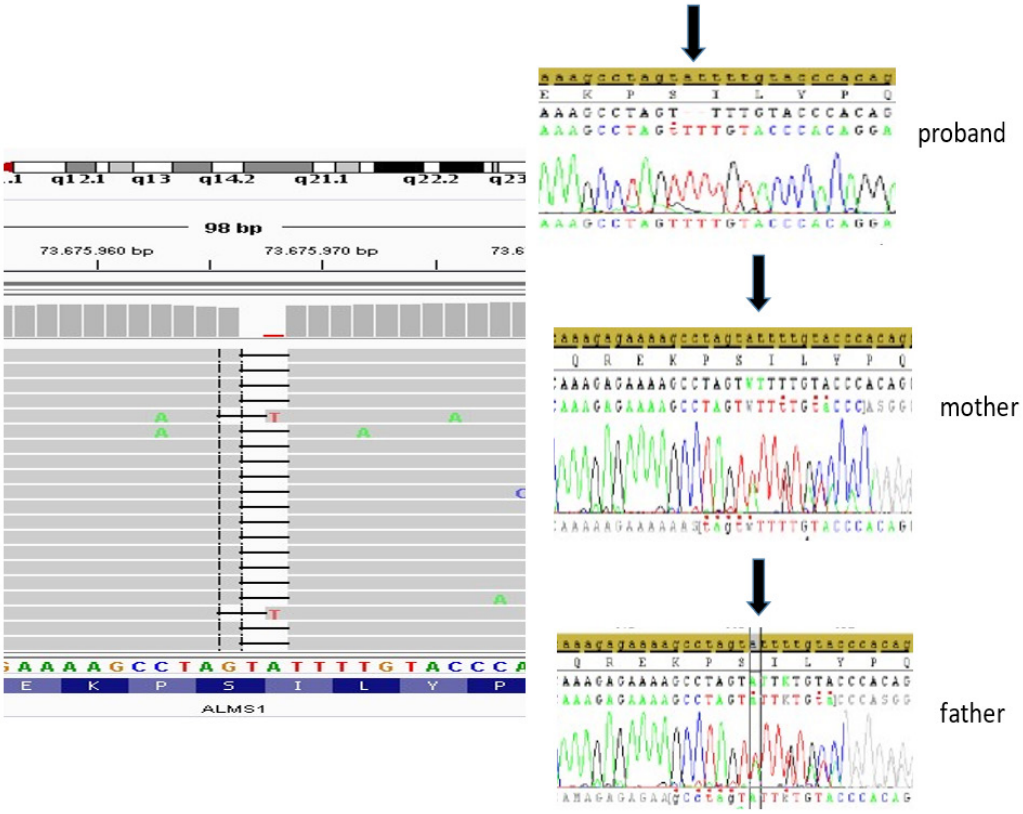

Figure 1. Next-generation sequencing (NGS) analysis result of the proband, proband's mother and proband's father.

to the abnormality in gonadotropin values, the patient underwent an LH-RH stimulation test. Accordingly, the patient was followed up in terms of precocious puberty after LH value was 1.34 mIU/mL.

In addition, an oral glucose tolerance test (OGTT) was applied to the patient whose clinical and laboratory results showed signs of insulin resistance. As a result of the test, 0th-minute glucose was 73 mg/dL, at 1st hour 128, and at 2nd-hour glucose value was 108 mg/dL, and total insulin was 1,144 µIU/mL. Oral metformin treatment was started in the patient who was found to have insulin resistance. The patient whose postnatal hearing screening was normal, hearing impairment was first noticed at the age of 5 years. At that point she was diagnosed with bilateral sensorineural hearing loss (SNHL) by audiometry test and was given a pair of hearing aids. An echocardiogram and abdominopelvic USG were performed on the patient, Echocardiogram was normal, however, USG showed minimal hepatosplenomegaly with grade 1 hepatic steatosis. At five years of age due to learning difficulties the Denver developmental screening test was performed on the patient. The result showed a neuromotor developmental delay in the patient and was found to be compatible with three years of age. Therefore, a brain magnetic resonance imaging (MRI) and electroencephalogram (EEG) were obtained from the patient. MRI showed evident central and peripheral cerebrospinal fluid distances, indicating a cortical atrophy, and EEG showed the presence of a background rhythm irregularity in cerebral bioelectric activity.

The proband was followed for years with a pre-diagnosis of Bardet-Biedl syndrome, however after the patient's clinical examination, we applied the clinical exome sequencing (CES) panel with the preliminary diagnosis of diseases that cause syndromic obesity such as Bardet-Biedl syndrome and Alström syndrome. As a result of the analysis, we detected a homozygous deletion

in two nucleotides in the *ALMS1* gene (c.2311_2312del). The mentioned deletion causes a frameshift mutation resulting in a premature termination codon (p.Ile771PhefsTer13) that causes a structural defect in the ALMS1 protein which leads to a malfuncting protein (Figure 1). We then applied Sanger sequencing both to confirm the result and to determine the carrier status of the parents. It was confirmed by Sanger sequencing that both parents were heterozygous for the same variant (Figure 1).

In this study, we identified a homozygous mutation of the *ALMS1* gene, and to the best of our knowledge, this variant has not previously been reported as homozygous in the literature (The Genome Aggregation Database (gnomAD), Leiden Open Variation Database (LOVD), and Pubmed). We hope by reporting this mutation we can expand our knowledge and spectrum of *ALMS1* variants in AS.

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Letter

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Atrial invasion from primary lung adenocarcinoma extension *via* the pulmonary vein

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SUMMARY Intravascular extension of lung adenocarcinoma is one of the four defined routes of metastasis to the heart but is rarely described in the literature. This is a rare case of primary lung adenocarcinoma with intravenous extension to the left atrium via the pulmonary vein. A 56-year-old female presented to the hospital with chest tightness and dyspnea. Chest computed tomography revealed a right hilar mass extending through the right superior pulmonary vein into the left atrium. Transthoracic echocardiography revealed a large, partially mobile left atrial mass occupying the entire atrial cavity and affecting mitral valve closure. Endobronchial ultrasound with transbronchial biopsy of the right middle lobe of the lung histologically showed a poorly differentiated adenocarcinoma compatible with the primary lung cancer. The patient was deemed a poor surgical candidate by cardiothoracic surgery due to the extent of metastasis and was started on chemoradiation. The patient's left atrial tumor mass started shrinking in size after starting the treatment. This unique case displaying intravascular extension of lung cancer to the left atrium has rarely been described in the literature.

Keywords lung adenocarcinoma, pulmonary vein, left atrium, invasion

The literature has rarely reported direct intravenous extension of non-small cell lung cancer to the heart (*1*). Involvement of the mediastinum in lung cancer is associated with an extremely poor prognosis, and many surgeons consider such tumors inoperable. Choosing the management between surgical resection, radiation, and conservative management continues to be challenging. We discuss the case of a patient presenting with the left atrial invasion of primary lung adenocarcinoma *via* the pulmonary vein, along with a brief review of the literature. Informed consent was obtained from the patient, and this study was approved by the institutional review board.

A 56-year-old female with a past medical history of resected right upper lobe lung granuloma and hypertension was admitted to our hospital with complaints of chest tightness and dyspnea that started 1 month before hospital admission. She reported progressive dyspnea over the month with an associated dry cough. Dyspnea was notably worse with exertion and in the supine position. Night sweats, fatigue, and recent weight loss were also reported. She had no fevers, chills, sputum production, or hemoptysis. A 60 pack-year smoking history was noted but quit 11 years prior. Upon admission, the patient was afebrile and hemodynamically stable. Oxygen saturation was 96% on room air. The electrocardiogram showed normal sinus rhythm with no ST or T wave changes. Chest X-ray (Figure 1A) was concerning for a right hilar mass. Follow-up chest computed tomography (Figure 1B and 1C) revealed a large left atrial mass extending into the right lung hilum through the right superior pulmonary vein with mild to moderate narrowing of the superior vena cava. Subsequent transthoracic echocardiography (Figure 1D) showed a large, partially mobile left atrial mass measuring 6.42 cm by 4.85 cm, occupying the entire atrial cavity and affecting mitral valve closure. The right ventricular systolic pressure was also noted to be 76.3 mmHg. The ensuing endobronchial ultrasound with transbronchial biopsy of the right middle lobe of the lung was completed. Histopathology showed poorly-differentiated adenocarcinoma compatible with primary lung cancer. Fluorodeoxyglucose-positron emission tomography scan showed large left atrial mass extension to the right hilum within the right superior pulmonary vein, with increased contrast uptake values in the left atrial and hilar component, suggesting malignancy. Cardiothoracic surgery was consulted. A nonsurgical

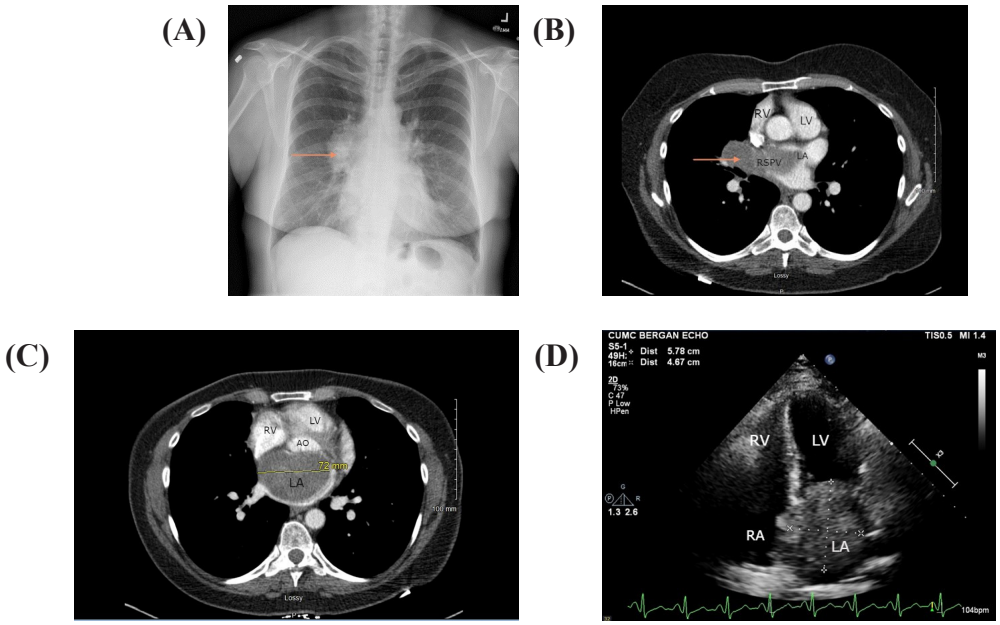

Figure 1. (A) Chest X-ray posteroanterior view showing a right hilar mass/adenopathy; **(B)** CT chest showing right hilar mass (arrow) extending through the right superior pulmonary vein into the left atrium; **(C)** CT chest showing large left atrial mass (dotted line); **(D)** Transthoracic Echocardiogram apical 4 chamber view, the dotted line showing the size of large mass noted in the left atrium. LA: left atrium; RA: right atrium; LV: left ventricle; RV: right ventricle; RSPV: right superior pulmonary vein.

management approach was opted due to the extent and degree of tumor spread. The patient was started on chemotherapy with carboplatin and paclitaxel, along with radiation therapy. After reviewing the PACIFIC trial (*2*), which showed improved survival in stage III lung cancer patients treated with chemoradiation followed by durvalumab, the patient was started on durvalumab. Repeat echocardiograms at 2 months and 3 months after discharge showed improvement in the size of the atrial mass. The patient tolerated the treatment very well and is back to working full time. She denied any cardiac or respiratory symptoms during the most recent follow-up at the cardio-oncology clinic 3 months after discharge.

Secondary cardiac tumors are the most common form of malignancy involving the heart. The incidence was previously thought to be very low, but with advancements in diagnostic modalities have risen significantly (*3*). Lung cancer has been described as the highest incident cause of secondary cardiac malignancy followed by breast cancer, malignant melanoma, and leukemia (*3*). Cardiac metastasis has been described to have three main routes of spread to the heart: distant (lymphatic/hematogenous spread), direct invasion, and intravenous extension; with the major metastatic pathway from the lungs described as a direct invasion (*3*). Atrial invasion of lung cancer through intravascular penetration, as described above, is extremely rare.

In a majority of cases such as the one described here, symptoms of lung cancer or heart failure are the presenting complaints (*4*). Obstruction of the pulmonary veins or mitral valve orifice may also lead to pulmonary edema (*5*). Like atrial myxoma, tumors invading the left atrium may occupy a majority of the atrial cavity and can lead to mitral valve stenosis by prolapsing into the valve orifice, as seen in the described case. Thromboembolic events have also rarely been reported (*1,5*).

Initial imaging with chest computed tomography (CT) is often ordered for symptom evaluation. Transthoracic Echocardiography can provide essential information about mass size, location, mobility, and hemodynamic effects (*6*). However, it has several limitations including the restricted field of view, limited imaging of the extracardiac, and mediastinal structures. Transesophageal echocardiography offers additional imaging planes but is more invasive (*7*). Cardiac CT is a commonly used second-line diagnostic method that offers high-quality images with a greater spatial resolution (*8*). Positron emission tomography can also be used to identify cardiac masses; however, its availability remains limited. CMR imaging is often used for its strong tissue characterization with high-contrast resolution (*9*). Limitations of CMR include their contraindication in patients with intracardiac defibrillators and pacemakers.

Lung cancer involving the intrapericardial pulmonary veins or left atrium is classified as T4 (according to American Joint Committee on Cancer 8th edition) irrespective of the magnitude of tumor infiltration, and T4N2 in the setting of mediastinal lymph node metastasis as in the present case. Initial management should include a surgical evaluation. When appropriate, the preference is complete tumor resection in combination with chemotherapy and/or radiotherapy (*4*). Literature review showed reports mostly describing partial left atrium resection without cardiopulmonary bypass; however, this is only possible if the wall of the left atrium is infiltrated locally without atrial cavity involvement (*10*).

Perioperative mortality for this procedure is reported at 5 to 18% (*7*). The 5-year survival rate in cases of non-small cell lung carcinoma penetrating the left atrium ranges from 0% to 22%. Factors associated with unfavorable effects on survival include mediastinal lymph node involvement (stage N2), location of the primary tumor in the lower lung lobes, and incomplete surgical resection (*10*). In our case, surgical resection was deferred due to extent of cancer involving the mediastinum and major blood vessels. The patient significantly benefited from the chemoradiation after discharge.

In conclusion, if the tumor is in an intra-atrial position or extensively infiltrates the left atrial wall, radical resection under cardiopulmonary bypass is recommended, however, if mediastinal lymph nodes are involved, patients have a poor prognosis and should be treated conservatively.

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Letter

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Differential diagnosis in Rosai-Dorfman disease: A rare case of isolated hepatic presentation mimicking a metastatic tumor with positive 18-FDG uptake

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SUMMARY Rosai-Dorfman disease (RDD) is also called sinus histiocytosis with massive lymphadenopathy, and it is caused by a histiocytic disorder with unclear etiology. It usually involves cervical lymph nodes, but it may also present with extranodal involvement. We report a rare condition of isolated hepatic RDD without nodal involvement, clinically manifested with three-month abdominal pain and tenderness of the right hypochondrium. CT- and PET-CT scans were compatible with a secondary lesion from an unknown primary tumor. Therefore, the patient underwent an atypical liver resection. Immunohistochemistry and histological results were compatible with a diagnosis of RDD. RDD is characterized by phenomena of emperipolesis, histiocytic proliferation and positive immunostaining for CD14, CD68 and S-100 protein. Cases of isolated gastrointestinal localization of RDD are particularly rare, especially in the liver. Instrumental exams might confuse RDD with other malignancies. RDD is a rare entity, which might be misdiagnosed using PET-CT due to its similarities with malignant tumors. An accurate multidisciplinary approach may help to clear diagnostic clues of this uncommon disease.

Keywords Rosai-Dorfman disease, sinus histiocytosis, liver surgery, hepatectomy

Sinus histiocytosis or non-Langerhans cell histiocytosis, also known as Rosai-Dorfman disease (RDD), represents a rare macrophage-related disorder of uncertain etiology (*1*). RDD was determined as a specific clinicopathological entity by Rosai and Dorfman in 1969 (*2*).

RDD typically develops as a localized or disseminated extranodal disease, triggering emperipolesis and histiocytic proliferation in multiple organs (*1*). It presents with fever and leukocytosis, associated with a massive and painless cervical lymphadenopathy. Immunohistochemistry (IHC) staining is often positive for S-100 protein, CD14, CD68, CD163 and negative for CD1a staining (*3*).

Gastrointestinal (GI) localizations of RDD are exceptional. A few sporadic cases with isolated hepatic involvement are reported in the literature (*3-5*). We herein describe a rare case of hepatic RDD, presenting with multiple liver nodules without nodal involvement and the role of 18-FDG PET-CT scans.

A 27-year-old Caucasian male presented with a three-month chronic nocturnal sweating and abdominal pain increasing with exertion. The patient was originally followed by another institution and dismissed with a diagnosis of liver metastasis. Nevertheless, a primary tumor could not be recognized even after a liver biopsy, which did not report any malignant cells. After being dismissed, the patient came to our attention complaining with the same symptoms. The physical examination noticed a mild tenderness of the right hypochondriac region with no other major symptoms. Laboratory tests showed C-reactive protein (CRP) levels of 21.5 mg/L. All oncological markers, including Chromogranin A tested negative. The abdominal ultrasound (US) described three hepatic nodules in VI, VIII and II-III segments (maximum diameter: 30 × 28 mm). An abdominal computed tomography (CT) with contrast was executed, showing three focal hypodense hepatic lesions, with low marginal enhancement and blurred borders, mimicking a metastatic lesion (Figure 1A). A background check on the genetic family history resulted in being

negative for chronic liver diseases, viral hepatitis and malignancies. Thus, a pancolonoscopy and a gastroscopy were performed to detect possible primary lesions, but no clues for primary gastrointestinal malignancies were identified. A contrast-enhanced US (CEUS) was performed. It showed an early and peripheral nodular contrastographic wash-out, typical of secondary lesions, also confirmed after a contrast-enhanced MR cholangiography (MRCP). Specimens from the liver nodules were collected for histological purposes. The positron emission tomography (PET)-CT scan showed an intense 18-fluorodeoxyglucose (FDG) uptake by the hepatic nodules (Figure 1B). As a consequence, an elective surgical intervention was planned. An "open approach" was chosen since the diagnosis was uncertain. The abdominal inspection was negative for suspicious malignancies, while the intraoperative US confirmed the lesions. The intraoperative extemporary pathologic exam resulted in being negative for malignant lesions and a wedge liver resection of the nodules was successfully performed (Figure 1C). The final pathologic report observed multinucleated histiocytic proliferation with emperipolesis. IHC staining detected a positive CD68 and S-100 protein with a negative CD1a. The postoperative recovery period was uneventful. The patient was successfully discharged on the VI post-operative day (POD). Three months after surgery, the patient presented at follow-up in an overall good condition.

RDD is defined as an uncommon non-Langerhans cell reactive histiocytic disorder (*2*). Globally, the prevalence of RDD accounts for one out of every 200,000 cases. The etiology of RDD is still under debate. Some studies attested to a role of viral infections from parvovirus B19, herpesvirus (HHV) and Epstein-Barr virus (EBV) (*1,5*). Other research did not endorse these results, but suggested an involvement of Kupffer cells in RDD with hepatic localization (*5*).

RDD mostly affects patients in the second and third decades of life, in otherwise good health condition with different symptoms that could mimic a lymphoma, such as a painless cervical lymphadenopathy, weight loss, night sweats, fever, neutrophilia, leukocytosis, anemia, lymphopenia and polyclonal hyperglobulinemia (*1,5*).

Histological features of RDD are emperipolesis, a massive sinusoidal dilation of large histiocytes with an abundant pale eosinophilic cytoplasm and a prominent nucleus (*1,5*). Also, RDD histiocytes test positive to CD14, CD68, CD163 and S-100 protein (*3*). Therefore, the RDD diagnosis mainly relies on histology, IHC and diagnostic imaging assessments.

Despite the fact that an RDD extranodal involvement might be encountered in more than 40% of patients with no lymphadenopathy, an intra-abdominal involvement is reported in around 4% of RDD literature cases (*3*). Gastrointestinal localizations of RDD accounts for < 1% of reported cases, representing an occasional finding,

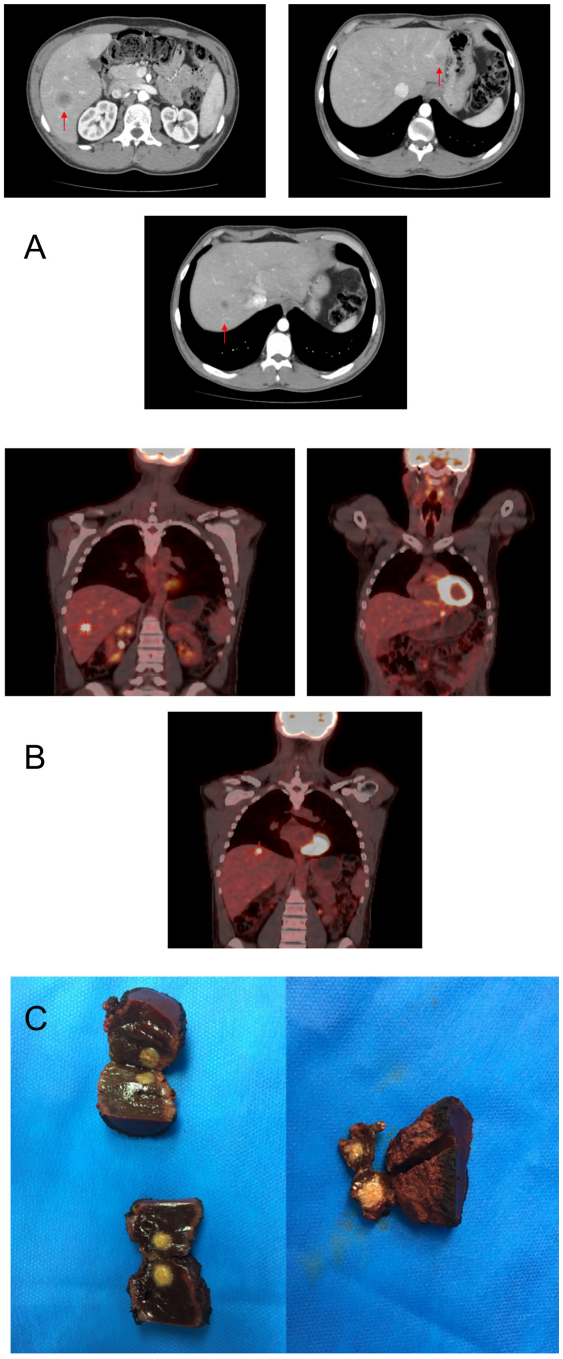

Figure 1. (A) Computed tomography (CT) scan showing an axial view of the hepatic lesions, presenting as non-enhancing hypodense nodules; (B) Hepatic nodules showing intense FDG uptake after PET-CT scans; (C) Liver parenchyma surrounding the three hepatic lesions removed.

particularly when diagnosed in liver and pancreatic tissues (*3,5*). Overall, a systemic RDD could involve the hepatic parenchyma, but reports about isolated liver lesions are extremely limited, to date (*3*).

Like other lymphoproliferative disorders, RDD lesions are FDG-avid, especially in the extranodal areas (*5,6*). Hence, despite an intense FDG uptake, which deceived an hepatic malignancy, the results of PET-CT should be carefully interpreted. In fact, in this specific

case the FDG avidity can be attributed to the intrinsic inflammatory and infiltrative component of the RDD process, and it must be remembered that a positive PET-CT is not always linked to malignant conditions (5,7). Moreover, in our case, the combination of a non-diagnostic biopsic report for tumoral cells, negative biochemical tumoral markers and the young age of the patient should have driven the diagnosis to a different etiology of pathology. On the other hand, the rarity of RDD and the absence of clear univocal guidelines concerning RDD did not facilitate its identification.

Steroids represent the first-line therapeutic option in symptomatic extensive RDD, and they are recommended with a systemic symptomatic RDD or when vital organs are threatened (*1*). In our case, a liver resection was performed to clear a diagnostic dilemma. Cases with airways, orbital and central nervous system involvement might benefit from radiotherapy, despite no clear guidelines available (*1,8*). Refractory cases to surgery and other treatments should consider chemotherapic regimens, which offer contrasting rates of success (*7,9*).

To date, this is the first young adult patient reported with multiple and solitary hepatic RDD lesions, without lymphadenopathy. The peculiarity of this case also relies on the role of PET-CT scans in RDD differential diagnosis, which could easily deceive the diagnosis of liver malignancies. Hence, according to our experience, IHC and histopathological exams are still crucial for RDD diagnosis. Clear guidelines concerning RDD are still lacking, and a multidisciplinary approach is paramount to promptly identify the correct diagnosis.

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Letter

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Granulomatosis with polyangiitis in gingiva: A rare case of isolated presentation

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SUMMARY Granulomatosis with polyangiitis (GPA) is a rare autoimmune disease characterized by necrotising granulomatous inflammation of upper and lower respiratory tract, vasculitis and glomerulonephritis. This ailment may present with cough, haemoptysis, sinusitis, nasal deformity, skin lesions, malaise, fever, anorexia, and weight loss. Oral manifestation includes strawberry gingivitis, which is a pathognomonic clinical presentation. Here, we present a case of GPA in gingiva as the first manifestation. Clinical examination of the oral cavity revealed granular, erythematous gingival enlargement in the lower anterior teeth region involving papilla, marginal and attached gingiva with shiny and pebbled surface. Histopathological examination showed pseudoepitheliomatous hyperplasia with vasculitis and inflammation in the connective tissue, neutrophilic infiltration and abscess formation with haemorrhage were noted. Laboratory investigations revealed Proteinase 3 (PR3) antigen and Glomerular basement membrane (GBM) antigen were positive. Clinical, histopathological and laboratory investigations enabled the diagnosis of Granulomatosis with Polyangiitis. We present this rare case report of GPA with primary manifestation in gingiva.

Keywords granulomatosis with polyangiitis, Wegener's granulomatosis, strawberry gingivitis

Wegener's granulomatosis, currently known as Granulomatosis with Polyangiitis was initially described by the German pathologist Friedrick Wegener in 1936 (*1*). This is a rare, systemic disease characterised by necrotising granulomatous inflammation of the upper and lower respiratory tract, vasculitis and glomerulonephritis (*2*). Despite years of research, the aetiology of this disease remains unknown (*2*).

The disease can present in either localised or generalised form. Localised form can involve predominantly upper respiratory tract without any vital organ involvement (*3*). In around 6-13% of cases, lesions in the oral cavity are reported. It is noteworthy that, oral cavity as initial site of presentation was noted only in 2% of the cases (*1*) Clinical manifestations vary from patient to patient, thus forming a challenge in prompt diagnosis and treatment. Rapid progression with multi-organ involvement can be potentially fatal (*4*).

Here, we present an extremely rare case of granulomatosis with polyangiitis of gingiva as the first site of occurrence without any systemic involvement.

A 32-year-old male patient reported to the Department of Periodontology, Krishnadevaraya College of Dental Sciences and Hospital, Bengaluru with a complaint of painful gingival swelling with intermittent bleeding for the past 2-3-months. The patient had visited a general dental practitioner and underwent scaling & root planning followed by administration of medication (Clotrimazole oral paint, Chlorhexidine mouthwash, Analgesics – Aceclofenac (325 mg) + Paracetamol (100 mg), Antibiotic - Amoxicillin 500 mg for 5 days). Respiratory tract infections were elicited and found to be negative. Similarly, there were no abnormalities detected in relation to eyes and kidney. Patient had the habit of smoking cigarettes (0.9 pack years), however, he reports to have quit the habit for the last 2 years. Patient also reported habit of pan chewing with tobacco for 6 years and has quit in the past 2 months. Oral examination revealed a purplish red, irregular, diffuse erythematous enlargement involving interdental papilla, marginal and attached gingiva (Bokenhamp Grade 3 gingival enlargement) with granular, shiny and strawberry like surface, in the lower anterior teeth region (#33-#43). The lesion was tender, soft and oedematous in consistency, immobile in attached gingival region and mobile in the papillary and marginal gingiva with sessile attachment

to underlying tissues and presented with irregular edges. Bleeding was present on probing the involved region. Mobility of teeth was absent and alteration of colour and surface texture was noted in papilla of upper canine region. No discharge and alteration of taste was seen (Figure 1A). Radiographic evaluation (orthopantmography and Chest X-ray) showed no abnormalities (Figure 1B). Routine blood investigation showed increased levels of Erythrocyte Sedimentation Rate (ESR) – 55mm/hr. C-Reactive protein was normal. The anti-neutrophil cytoplasmic antibody (ANCA) analysis revealed, myeloperoxidase (MPO, p-ANCA) as negative and Proteinase 3 (PR3) antigen (c-ANCA) and Glomerular basement membrane (GBM) antigen as positive. Informed and written consent was obtained from the patient.

An incisional biopsy was performed, and histologic examination demonstrated predominantly parakeratinised stratified squamous epithelium exhibiting pseudoepitheliomatus hyperplasia, spongiosis, acanthosis and formation of neutrophilic abscess (Monroe's abscess) within the epithelium. The underlying connective tissue was oedematous and consisted of dense mixed inflammatory cell infiltrate consisting of neutrophils, eosinophils, lymphocytes, plasma cells, macrophages, and mast cells. Proliferating dilated blood vessels, a few congested blood capillaries, areas of haemorrhage, and extravasated red blood cells were evident (Figures 2). Considering the clinical, radiographic, histological and laboratory findings, a diagnosis of GPA was made. The patient was referred to a centre for immunotherapeutics for further management (Supplemental Table S1, *http://www.irdrjournal.com/action/getSupplementalData.php?ID=97*).

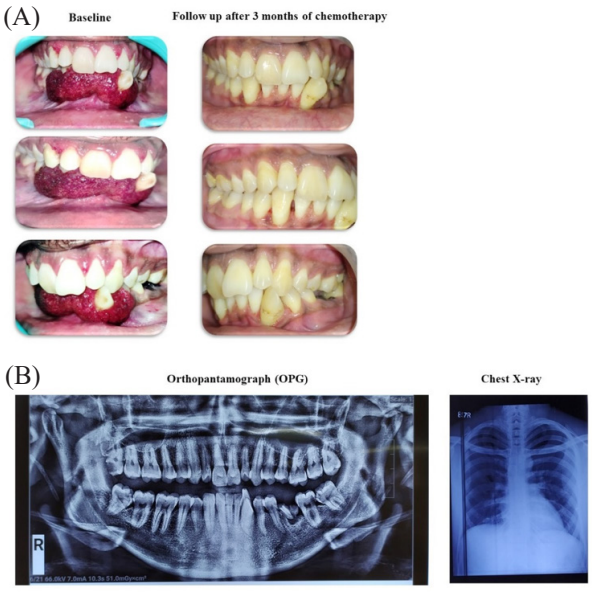

Figure 1. (A) Clinical photographs at baseline and 6-month follow up; (B) Orthopantamograph (OPG) and chest x-ray showing no abnormalities.

The etiology of GPA remains unclear, while various environmental factors like exposure to cadmium, silica, mercury, sand dust, and volatile hydrocarbons have been studied as possible causative agents (5). Furthermore, bacterial and viral agents like Staphylococcus aureus, hepatitis C virus, Epstein-Barr virus, and cytomegalovirus have also been implicated in modulating the clinical phenotype of the disease. Besides, certain medications – phenytoin, hydralazine, anti-thyroid medications, sulfasalazine and allopurinol have been noted in previous reports (*6*). Lately, Fiona *et al*. provided insight into former smoking being associated with development of GPA with an odds ratio of 1.5, when compared with never smokers (*7*). Similarly, McDermott *et al*. have reported association between ANCA associated vasculitis (AAV) and cigarette smoking, suggesting a possible mechanism between respiratory exposure and development of AAV (*8*). A notable fact is that, the patient presented in this case was a former smoker for 6 years (0.9 pack years). Cigarette smoking is a modifiable risk factor which should be given due importance.

GPA affects lower and upper respiratory tracts, thus patients complain of sinus congestion, nasal obstruction, epistaxis, otitis media, or dyspnoea. None of these symptoms were reported by the patient in this case. Oral manifestations of GPA include ulcers of the palate, tongue or other areas of oral mucosa which show delayed healing, oro-nasal fistulas, mobility, loss of teeth and nodules, and swelling of lips. Very rarely parotitis (inflammation of the sublingual salivary gland),

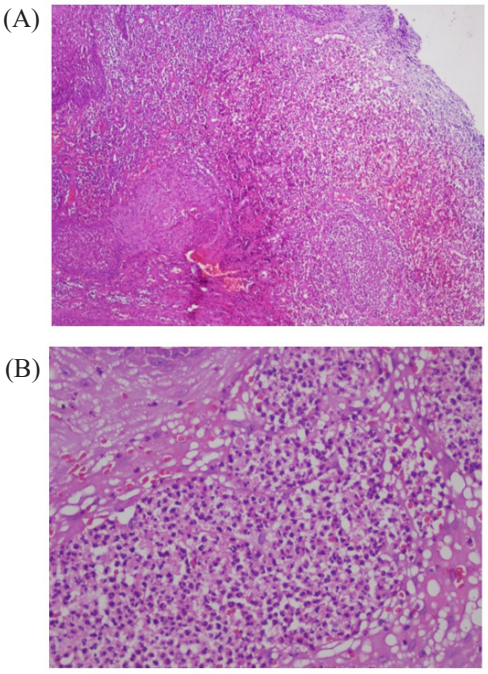

Figure 2. (A) Hematoxylin and eosin (H&E) section showing pseudoepitheliomatous hyperplasia with vasculitis and inflammation in the connective tissue (20×); (B) Hematoxylin and eosin (H&E) section showing neutrophilic infiltration and abscess formation with haemorrhage (40×).

which is painful, is also reported. In the present case, manifestation was restricted only to gingiva, showing the classical picture of "strawberry gingiva" (*9*).

When GPA is suspected due to history and clinical examination of patient, measurement of complete blood count, erythrocyte sedimentation rate, C-reactive protein, proteinuria, urine analysis, serum creatinine, and blood urea nitrogen levels is recommended (*10*). In the current case, only ESR was elevated; the C-reactive protein (CRP) and urine analysis showed no significant deviations. The current recommendations on ANCA testing requires screening of two main patterns of ANCA: cytoplasmic pattern (c-ANCA) and perinuclear pattern (p-ANCA). Studies have shown that c-ANCA is 80-100% specific for GPA (*4*). In the present case, c-ANCA along with glomerular basement membrane antigen were found to be positive. The clinical picture of hyperplastic erythematous gingival tissue may give rise to differential diagnoses like drug induced gingival enlargement, plasma cell gingivitis, sarcoidosis, leukemic enlargement, and chronic inflammatory enlargement. However, the classic clinical presentation and further serological tests helped in ruling out the differentials and confirming the diagnosis to GPA. Sometimes even histopathological findings alone maybe inconclusive, hence it is mandatory to correlate with clinical and systemic findings to arrive at a diagnosis. Special staining to rule out bacterial or fungal etiology is also recommended.

Various treatment protocols have been reported, which include cyclophosphamide and azothiopurine as the main drug of choice. Also, Glucocorticoids with methotrexate are used to induce remission of GPA. A study conducted at National Institute of Health, a regimen of cyclophosphamide (2 mg/kg body weight per day) with prednisone (1 mg/kg body weight), showed complete remission in 93% of the patients (*3*). Currently, Rituximab (monoclonal antibody against CD20) has also been approved as a therapy. In the reported case, a combination of mycophenolate (500 mg, 1-0-2), methotrexate (15 mg, 1/week), hydroxychloroquine (200 mg, 1-0-1), and prednisone (30 mg – 20 mg – 10 mg for 2 weeks and 30 mg – 20 mg for further weeks) was given in first phase up to 3 months. At the end of 3 months, complete resolution of oral lesion and strawberry gingiva was noted. Following this, the dosage was tapered. When prompt diagnosis and correct therapeutic decisions are made, resolution of lesions occurs within a few weeks. However, with tapering of immunosuppressive therapy there are chances of relapse of the disease.

When oral manifestation of GPA is present, albeit very rare, it is imperative for a dental practitioner to critically analyze the clinical, systemic, hematological, and histopathological manifestations to enable an early and accurate diagnosis and appropriate referral for medical management. There may be an isolated clinical presentation of GPA in gingiva as in the current case, which affirms that health professionals should be acquainted with oral manifestations of diseases.

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IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research (Print ISSN 2186-3644, Online ISSN 2186-361X) is an international peer-reviewed journal. Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables. Supplementary Data are permitted but should be limited to information that is not essential to the general understanding of the research presented in the main text, such as unaltered blots and source data as well as other file types.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of a maximum of 10 figures and/or tables and 100 references. Mini reviews are also accepted, which should not exceed 4,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 50 references.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in *Intractable & Rare Diseases Research* in the last 6 months or issues of general interest. Summaries of research results and sharing of experiences in clinical practice and basic research (findings based on case reports, clinical pictures, *etc.*) can also be published as Letters. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

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For publishing and ethical standards, *Intractable & Rare Diseases Research* follows the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (*http://www.icmje.org/recommendations*) issued by the International Committee of Medical Journal Editors (ICMJE), and the Principles of Transparency and Best Practice in Scholarly Publishing (*https://doaj.org/bestpractice*) jointly issued by the Committee on Publication Ethics (COPE), the Directory of Open Access Journals (DOAJ), the Open Access Scholarly Publishers Association (OASPA), and the World Association of Medical Editors (WAME).

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4. Manuscript Preparation

Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *http://www.ICMJE.org*.

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For articles that are Original Articles, Brief Reports, Reviews, or Policy Forum articles, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For Communications, Editorials, News, or Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Three to six key words or phrases that do not occur in the title should be included in the Abstract page.

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Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. The EndNote Style of *Intractable & Rare Diseases Research* could be downloaded at **EndNote** (*https://www.irdrjournal.com/examples/Intractable_Rare_Diseases_Research.ens*).

Examples are given below:

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Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):

Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

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Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

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World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

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