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Metastatic Malignancy Presenting as Thrombotic Thrombocytopenia Purpura; Report of Two Cases

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Abstract:
Background
Cancer associated thrombotic microangiopathy (CA-TMA) is a rare diagnosis that while confronting a patient with evidence of microangiopathic hemolytic anemia and thrombocytopenia with normal ADAMTS13 enzyme activity, should be considered. Here we present two cases of cancer associated TMA; breast cancer and gastric cancer.
Case presentation
Case 1 was a 40-year-old man presented with abdominal pain, icterus and weight loss and laboratory tests revealed microangiopathic hemolytic anemia. He received 6 sessions of plasma exchange under diagnosis of thrombotic thrombocytopenic purpura. Bone marrow biopsy and immunohistochemistry revealed clusters of non-hematopoietic cells suggestive of gastrointestinal adenocarcinoma. Case 2 was a 51-year-old woman whose clinical history and laboratory tests were similar to case 1 except for a breast mass along with axillary lymphadenopathy. Bone marrow examination revealed clusters of non-hematopoietic cells and core needle biopsy revealed invasive lobular carcinoma.
Both patients were unresponsive to plasma exchange and case 1 unfortunately shortly died after diagnosis but case 2 is alive and survived after receiving chemotherapy.
Conclusion
Cancer associated TMA can rarely be seen as the first manifestation of a malignancy and causes a diagnostic dilemma for clinicians.The prognosis of CA-TMA is generally poor and initiatingchemotherapy is the only reliable treatment option.

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy (TMA) which is divided into immune and hereditary based on ADAMTS 13 enzyme level. Hence, confronting a patient with evidence of microangiopathic hemolytic anemia and thrombocytopenia but with normal ADAMTS13 activity, other causes of TMA including cancer associated TMA (CA-TMA) should be considered. Since CA- TMA is a rare pathologic condition and is not responsive to plasma exchange therapy, it requires a broad and precise differential diagnosis to achieve the most appropriate treatment (1). Here we present two cases of cancer associated TMA; breast cancer and gastric cancer.

Case presentation

Case 1

A 40-year-old male presented with abdominal pain, generalized weakness and icterus. Past medical history and drug history were unremarkable except for a history of 10-kilogram weight loss in the past month. In physical examination, drowsiness and icterus were

noted otherwise other examination were normal. Primitive laboratory test revealed severe anemia associated with thrombocytopenia, elevated LDH and abnormal liver function tests as shown in table 1.

Peripheral blood smear showed marked increase in schistocyte count and polychromasia suggestive of microangiopathic hemolytic anemia (MAHA) (Figure 1, Panel A). Under diagnosis of thrombotic thrombocytopenic purpura (TTP), emergent plasmapheresis along with dexamethasone were started. After 6 sessions, due to lack of response to primitive therapy and normal ADAMTS13 level, bone marrow aspiration and biopsy was performed that revealed infiltration of clusters of non-hematopoietic cells suggestive of metastatic malignancy (Figure 2, Panel A). IHC study of bone marrow was positive for CK7 and negative for CK20, CDX2, P63 and TTF1 suggestive of metastatic poorly differentiated CK7 positive carcinoma; therefor, gastrointestinal (GI) cancer particularly gastric adenocarcinoma was considered.

Chest and abdominopelvic CT revealed mediastinal and para-aortic lymphadenopathy. Pelvic MRI showed diffuse hyperintense signal in bone marrow of iliac crests suggestive of metastatic malignancy. Patient was scheduled for upper GI endoscopy and total colonoscopy but due to decreased level of consciousness and severe thrombocytopenia could not be performed and patient died shortly after diagnosis.

Case 2

The patient was a 51-year-old woman who presented with weakness and drowsiness and past medical history and drug history were unremarkable. On physical examination, lumps in the left and right breasts of about 2 cm along with lymphadenopathy in the left axillary area were noted. As mentioned in case 1, primitive lab tests revealed anemia and thrombocytopenia with increased LDH (Table 1) while peripheral blood smear demonstrated 5% schistocytes and 2% nucleated RBC; findings that were suggestive of TMA.

Daily plasmapheresis was started but the response was disappointing by the end of 10 days. The LDH and nucleated RBC count were rising and platelet count and Hb reduced while ADAMS13 activity and antibody were normal. Bone marrow aspiration and biopsy revealed cluster of non-hematopoietic cells; therefor work-up for secondary causes of TTP was performed. After confirmation of the breast mass in ultrasound and mammography, core needle biopsy was done and IHC results of breast mass and BMB were in favor of invasive lobular carcinoma (ER=positive, PR= Positive, HER2=negative). Chest and abdominopelvic CT scan showed diffuse bone metastasis. After patient

Table 1. Laboratory results of patients.

Lab test	Case 1	Case 2	Lab test	Case 1	Case 2
WBC (10^3/UL)	3500	8400	AST (u/l)	55	64
Hb (g/dl)	7	7.5	ALT (u/l)	65	78
MCV (fL)	85	91	ALK.P (u/l)	672	477
MCH(Pgm)	31	30	Bilirubin total (mg/dl)	3	1.8
Platelet (X1000/mm^3)	25000	75000	Bilirubin direct (mg/dl)	0.4	0.2
LDH (u/l)	1600	5452	PTT (s)	30	33
Reticulocyte count (cells X 10^9/L)	5.5	3.5	INR	1	1.2

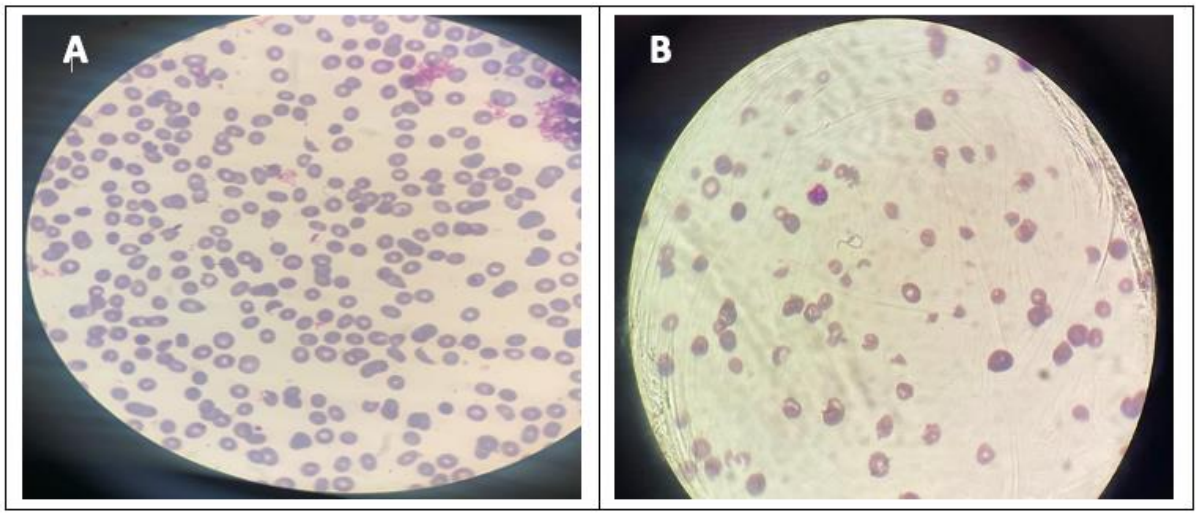

Fig 1. Peripheral blood smear patients revealed anemia and evidence of microangiopathic hemolytic anemia (Panel A, case 1; Panel B, Case2)

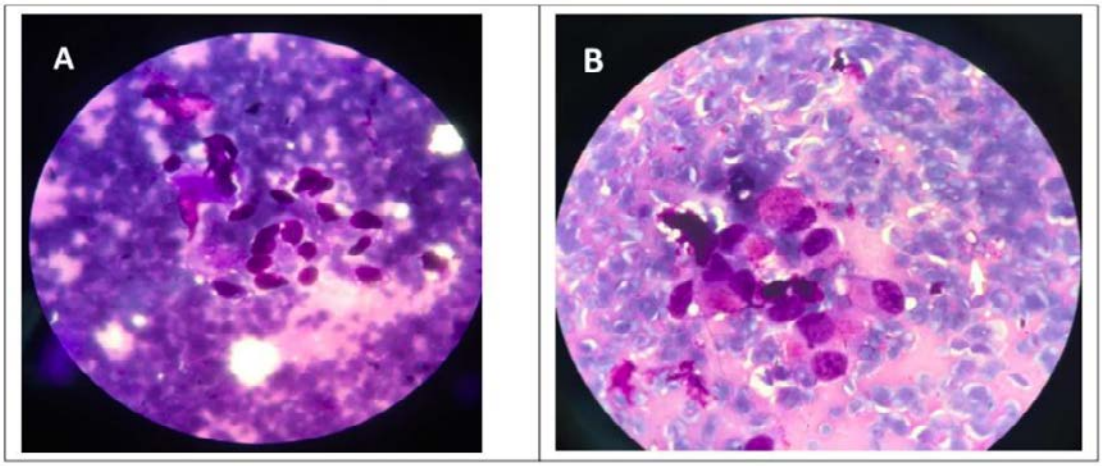

Fig 2. Bone Marrow aspiration revealed Clusters of non-hematopoietic cells in the marrow (Panel A, Case 1; Panel B, Case 2)

received the first course of AC regimen (Epirubicin and Cyclophosphamide) platelet count dramatically rose to ≥150000 and LDH was reduced and after 4 coarse the masses were 50 % decreased in size.

DISCUSSION

Cancer associated TMA (CA-TMA) can rarely be seen as the first manifestation of a malignancy and causes a diagnostic dilemma for clinicians. The most common malignancies associated with TMA are gastric, breast and prostate while lung and metastatic signet ring carcinoma of unknown origin have also been mentioned. The prognosis of CA-TMA is generally poor and initiating chemotherapy is the only reliable treatment option.

The exact pathogenesis of cancer associated TMA is unknown however some possible explanations are bone marrow infiltration with cancer and paraneoplastic syndrome (1). Other studies have suggested that fibrinoid necrosis of the bone marrow and tumor cell emboli could be other causes of CA- TMA while tumor-derived factors, procoagulants, and some chemotherapy agents are also considered causative agents of CA-TMA (2,3,4). The most common malignancies associated with TMA are gastric, breast and prostate (5) while lung and metastatic signet ring carcinoma of unknown origin have been mentioned in other studies. Signet ring carcinoma which is a subtype of mucin producing adenocarcinoma can arise from all organs but most common organs are stomach, breast and colon. Mucin probably has a direct detrimental effect on the endothelial cells leads to change their endothelial function and thus increases the production of ultra large von Willebrand factor multimers which could be another explanation of CA-TMA (6).

In comparison of idiopathic TTP with CA-TMA, a systematic review and report of ten patients based on Oklahoma TTP-HUS registry revealed that patients with malignancy are more often men with more frequent respiratory symptoms and longer duration of symptoms before diagnosis. Considering laboratory tests and treatment, patients with malignancy tend to have greater levels of LDH, less responding to plasma exchange and a higher mortality rate (7). Although ADAMTS13 activity level of less than 10 % could differentiate these two entities from each other but severe ADAMTS13 deficiency of less than 5 % is not common in both groups (7).

Although diagnosis of TTP and urgent treatment with plasma exchange is critical in patients presenting with MAHA but response to plasma exchange is limited in patients with CA-TMA (8). Plasma exchange could even be harmful in patients with malignancy because it may cause microcirculatory damage and this may enhance microthrombi formation (9). Furthermore, avoidance of unnecessary plasma exchange in these patients will decrease the additional risk of plasma exchange complications such as catheter infection, sepsis, venous thrombosis and pericardial tamponade (10). The ultimate treatment of CA-TMA is systemic chemotherapy, thus review of the literature revealed that despite initiating treatment early in the course of the disease, most patients die within few weeks and the most common cause of death was infection.

In conclusion, the prognosis of CA-TMA is generally poor and initiating chemotherapy is the only reliable treatment options.

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None

Conflict of interests
The authors declare no conflict of interest

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Mechanistic and Diagnostic Roles of Kallikrein Related Peptidases 2 (KLK2) in Prostate Cancer

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Protease inhibitors
Prostate cancer
Diagnostic marker of prostate cancer

Abstract:
Kallikrein related peptidases (KLKs) are a group of serine-like proteases such as chemo trypsin and trypsin, which are regulated by steroid hormones and play a vital role in a variety of natural and physiological functions through their proteolytic activity. However, involvement of these proteases has been reported in many pathological conditions, such as various types of malignancies. Deregulation of the expression of genes encoding kallikrein, including KLK2, is often associated with many types of cancer, in particular prostate cancer. This review provides an overview of the gene and protein structures and function of KLKs particularly, KLK2, at the molecular level, and also summarizes the role of KLK2 in the pathobiology of prostate cancer and the possible mechanisms involved in its progression. Finally, the importance of this protein is studied as a specific diagnostic marker along with PSA marker as well as therapeutic target of KLK2 in treatment of prostate cancer. A comprehensive understanding the structure and activity of this protein in prostate cancer can provide a valuable tool for future clinical practice that can be used to evaluate the clinical outcome and select the most appropriate treatment strategy. The critical role of KLK2 in promoting cell growth, migration, metastasis, angiogenesis and inhibiting apoptosis in prostate cancer cells, suggests KLK2 as the second diagnostic biomarker along with PSA with high specificity.

INTRODUCTION

Tissue kallikrein (KLK1) and kallikrein-related peptidase (KLK2-KLK15) are included of a subset of 15 chemotrypsin-like or trypsin-like serine protease encoded by a 265 kb multigene cluster family in chromosome 19q13.3-4 (Figure 1A) (1). The term of kallikrin refers to pancreas, firstly used in 1930 by Warl et al due to high concentration of the component in the pancreas. Firstly, kallikrin was identified as a proteolytic enzyme and named tissue kallikrin or glandular kallikrin/kallikrin 1 (KLK1) (2). Following this, kallikrein coding gene (KLK1) was discovered in 1985, and then two very structurally similar genes, KLK2 and KLK3 / prostate-specific antigen (PSA), were found in the same chromosomal region. After gene cloning and mapping, it was proposed that 14 of 15 genes of tissue kallikrein should be introduced as kallikrein-related peptidases (KLK2-KLK15) (3).

All KLK genes and proteins are identified by a series of similar structural and functional features, including the presence of: (Figure 1B) and (Figure 2) (4).

- Five coding exons with the same exon length and four intron regions with different length
- Conserved catalytic subunits, histidine (His), aspartate (Asp) and serine (Ser) in exons 1, 3 and 5, respectively.
- A number of spliced alternative copies
- A single peptide
- Amino acid sequence with 80-40% identity

In addition, the expression of KLKs is regulated by steroid hormones, and the proteolytic activity of KLK proteins is mediated by several ways, including zymogenic activation and complex formation with plasma and / or tissue inhibitors, whereas the inactivation of KLKs is mediated by cleavage and fragmentation. KLKs are expressed in a wide range of human tissues, mainly in the cytoplasm of glandular epithelial cells which are involved in a variety of natural and physiological functions, from regulating blood pressure to homeostasis and tissue regeneration as well as hormone processing (5). However, deregulation of KLK gene expression at the level of mRNA and / or protein in many types of cancer is

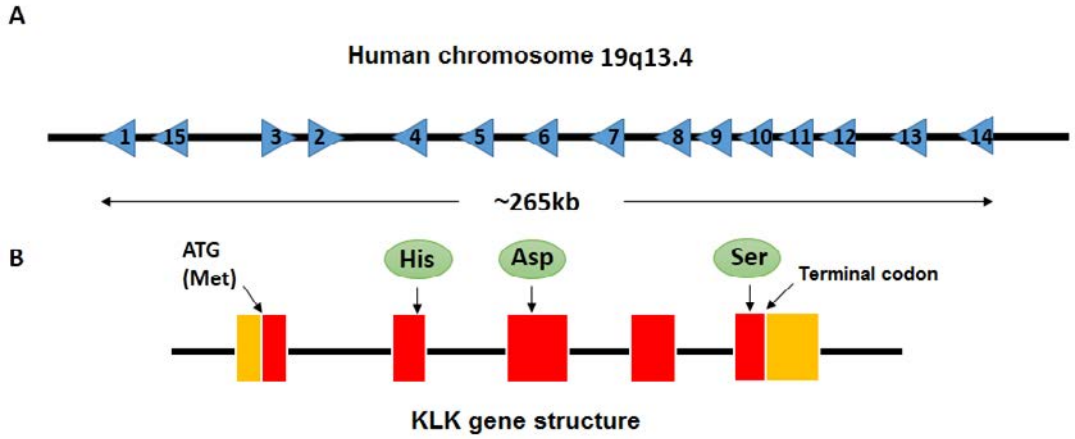

Fig1. A) The largest peptidase cluster in the human genome is related to kallikreins, B) The coding gene contains 5 exons separated by 4 introns.

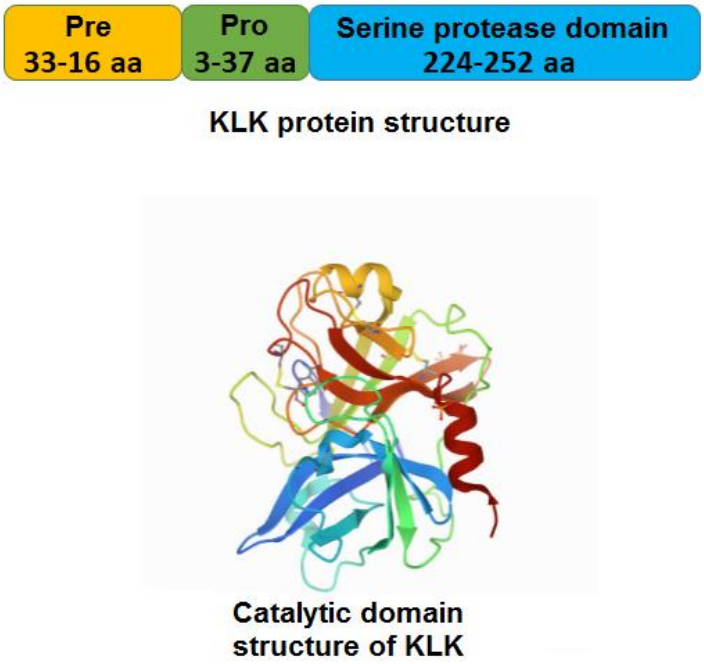

Fig 2. Linear and three-dimensional structure of KLK proteins (https://www.rcsb.org/)

often associated with the pathological profile of patients as well as using as a potential prognostic biomarker (4-7). The mechanisms influencing deregulation of KLK expression in cancers have not yet been fully clarified. However, a recent study suggests that chromosomal aberrations and changes in copy number of genes are responsible for the deregulation of KLK genes in many human malignancies. Numerous studies have provided evidence that KLKs are contributed to the development and progression of several cancer cells by modulating different factors and proteins (4, 7-9). They may stimulate or inhibit cancer cell growth through activating growth factors and interacting with other proteases. Furthermore, KLKs can mediate angiogenesis by activating and releasing angiogenic factors, and may also be involved in invasion and metastasis through proteolytic processing and degradation of extracellular matrix compounds (6, 7, 10).

The multiplicity and diverse role of these enzymes have introduced them as diagnostic markers and therapeutic targets in a large number of diseases such as cancer, nervous system disorders, skin diseases and diabetes (11-15). Interest in KLKs as cancer biomarkers began in the late 1970s to mid-1980s, followed by scientific groups that specifically elucidated KLKs, especially KLK3 or PSA, in malignant prostate gland in patients's serum (16, 17). Besides PSA, many other KLKs were proposed in screening, differential diagnosis and prognosis of prostate cancer that can be used as prostate cancer biomarkers in tissue or serum, providing valuable information about the prognosis of prostate cancer as well as rate of malignancy (1, 8, 17). This study provides a review on KLK2 as a member of the KLK family that plays a key role in the progression and diagnosis of prostate cancer. The role of KLK2 in the pathobiology of prostate cancer and the possible mechanisms involved in the progression of this cancer are summarized. Finally, this review presents

the importance of this protein as a specific diagnostic marker along with PSA marker in prostate cancer. Understanding the structure and activity of KLK2 and the mechanistic pathways implicated by KLK2 in the progression of prostate cancer, could offer the way for further studies to apply new treatment strategies as well as usage as a specific diagnostic marker in the diagnosis of prostate cancer.

Kalikrin-2 peptidase (KLK2)

Kalikrin-2 (KLK2) gene is a member of the KLK gene family and KLK2 protein is a trypsin-like serine protease which is highly expressed in prostate tissue. KLK2 is also called hK2. KLK2 is usually co-expressed with KLK3 or PSA and co-located in the prostate gland. KLK2 is about 1% of the total PSA concentration in seminal plasma and shares 80% homology with PSA or KLK3. Both KLK2 and KLK3 are highly expressed in prostate luminal epithelial cells and as secretary proteins are produced by prostate gland (18). Although expression of KLK2 may be observed in other organs, the level of expression in other tissues is much less than prostate and their biological activity remains unknown. Expression levels of KLK2 and KLK3 which are regulated by androgens, reflect the function and activity of the nuclear androgen receptor (AR) and their response to testosterone or other androgen hormones (19, 20).

KLK2 gene and protein

It has been hypothesized that the KLK2 precursor gene has been originated from the replication of the KLK1 gene, early in mammalian evolution. The KLK2 precursor is a non-functional pseudo gene in many mammals, including rodents, however it is highly expressed in prostate of other mammals whose expression of KLK2 is regulated by androgen. There is an enhancer containing androgen response element (ARE) in 5′UTR of transcription initiation site (21). KLK2 as a proto-oncogene can be regulated and overexpressed by androgens and androgen receptor signaling pathway in prostate cancer (22). KLK2 can also act as an androgen regulator, cooperating with the ARA70 regulatory protein to increase androgen activity and regulate cell growth during cancer development (23).

The identity of amino acid sequence between KLK1 and KLK2 or KLK3 (67-62%) is much greater than amino acid sequence of KLK4-15 (27-29%). Firstly, these proteases are produced as non-catalytic pre-proenzymes which require several post-translational modifications to form a catalytically active form. To obtain the active form of KLK2, a proteolytic cleavage in the signal sequence occurs by the signal peptidase, followed by a secondary breakdown with a trypsin-like peptidase enzyme, releasing a short peptide from the N-terminal region. Finally, the non-catalytic zymogen form is converted to the active single-stranded form of KLK2 with 237 amino acids. Substitution of alanine with valine 217 has shown a reduction in the catalytic efficiency of KLK2 (22, 24, 25). Figure 3 shows the three-dimensional structure of KLK2.

KLK2 activity and its role in the pathobiology of prostate cancer

Regulation of KLK2 activity is crucial for the maintenance of cell and tissue function and homeostasis, whereas KLK2 deregulation can be used as a cancer biomarker. Although there are some similarities between KLK2 and PSA in terms of their function, tissue expression and regulatory properties, KLK2 has different enzymatic activity compared to PSA. One of the most significant differences between KLK2 and PSA is that KLK2 is a trypsin-like serine protease, whereas PSA is a chymotrypsin-like one. KLK2 has a much higher catalytic activity than PSA and is able to activate either itself or PSA. Therefore, it suggests that KLK2 may also regulate biological activity of PSA (24-26).

KLK2 is secreted and activated into the seminal fluid. The gel-forming proteins (SEMG1 and SEMG2) are then proteolytically degraded by KLK2 to liquefy the seminal gel. This is an essential step for the release of sperm to the uterus in order to fertilize the ovum (21).

Moreover, KLK2 contributes to the normal

Fig3. KLK2 protein structure (https://www.rcsb.org/)

physiology of the prostate through hydrolyzing seminal vesicle proteins, hydrolyzing seminogelin I and II and fibronectin. Proteolytic cleavage of seminogelin and fibronectin by KLK2 indicates that this enzyme is also able to degrade cellular matrix proteins. This leads to liquefaction of the seminal clot and increases sperm motility (21, 22). However, KLK2 can also play a critical role in the pathological condition of the prostate gland. Research has also focused on the association between KLK2 and prostate cancer proliferation and invasion. Tumorigenicity activity of KLK2 is mediated by activating several molecules and proteins. KLK2 is able to inhibit the plasminogen activator urokinase inhibitor (uPAI) and activate uPA which is a protease initiating the proteolytic cascade, converting plasminogen to plasmin as well as degrading extracellular matrix proteins such as collagen types I and IV, fibronectin and laminin. It is contributed to tumor development and strongly associated with prostate cancer invasion and metastasis (22, 27, 28). Furthermore, KLK2 also has a vital role in activating matrix metalloproteases (MMPs) through proteolytic cleavage of pro-peptides (27, 28).

It has been reported that KLK2 may directly enhance the growth of cancer cells (10). Studies have shown that KLK2 is able to cleave insulin-like growth factor proteins and activate growth factor, and may also modulate the activity of parathyroid protein (PTHrP). Insulin-like growth factor (IGF) causes mitogenic and anti-apoptotic effects on normal and tumor cells through binding to IGF receptor. IGF availability and binding to IGF receptor can be regulated by IGF-binding proteins (IGFBPs). KLK2 is also able to degrade IGFBP2-5, thus leads to the high availability of IGF and may indirectly contribute to the proliferation and progression of cancer cells. High level of IGF in the blood and therefore high activity of the IGF pathway has been shown in prostate cancer (20-22). IGFBP3 can also induce apoptosis in prostate cancer cells. Therefore, KLK2-mediated degradation of IGFBP3 leads to inhibit apoptosis in these cells as well as increase in IGF availability which is implicated in proliferation and progression of prostate cancer cells. In addition, studies have shown that KLK2 plays a crucial role in promoting the growth and metastasis of prostate cancer cells by activating TGFβ (28-30).

The role of KLK2 in angiogenesis of prostate cancer cells is also mediated by proteolytic degradation of plasminogen and high molecular weight kininogen (HMWK). KLK2 cleaves HMWK proteolytically to activate and release bradykinin, a factor that induces smooth muscle cell contraction, which is implicated in formation of blood vessels in prostate tumors (28-30).

Another target of KLK2 is PARs (PAR1-4), a subtype of G protein-coupled receptors. Proteolytic activation of PAR1 is involved in regulating proliferation of prostate cancer as the increased expression of PARs has also been confirmed in prostate cancer. These proteins are activated by a proteolytic cleavage by KLK2 in the N-terminal domain (31). Besides of the physiological roles, PARs can interfere with the development of cancer-related signaling pathways such as MAP kinase and ERK, which promote cell proliferation and migration (Figure 4) (29, 30).

Table 1 presents the role of KLKs in particular KLK2 in prostate cancer.

Different forms of KLK2 and KLK3

There are various forms of PSA detected in the blood due to the catalytic activity of PSA and the existence of more than 10,000 times proteinase inhibitors in extracellular fluid. Once, enzymatically active PSA releases into the blood, can form irreversibly active complexes with extracellular protease inhibitors such

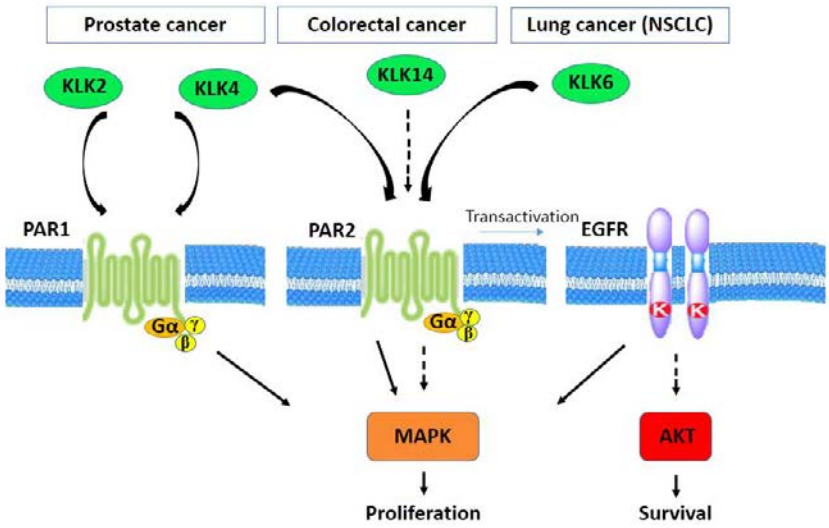

Fig 4. *The role of KLKs in cancer and specifically the role of KLK2 in activating PAR1 and activating the MAPK pathway in prostate cancer*

Table 1. An overview on the activity and role of KLKs, specifically KLK2, in prostate cancer

KLK	Target	Phenotype of cancer cell
KLK2-5, KLK11	IGFBP1-5 TGFβ1, 2 Growth hormones	Proliferation
KLK14 ،KLK2-4	IGFBP3 PAR-1	Fibroblast signaling
KLK2-3	HMWK Plasminogen	Endothelial signaling
KLK2-3	HMWK Plasminogen	Angiogenesis
KLK13-14 ،KLK2-5	Collagen type I, IV Fibronectin Laminin	EMT and migration
KLK13-14 ،KLK2-5	Collagen type I, IV Fibronectin Laminin Plasminogen	Invasion
KLK2-4	TGFβ1, 2 Plasminogen	Metastasis

as α1-antichemotrypsin (ACT or SERPINA3), α2-macroglobulin (A2M), pregnancyzone protein (PZP), α1-antitrypsin (SERPINA1) or protein inhibitor C (SERPINA5). Binding PSA to the inhibitors is the immune active dominant form of PSA in the blood (32, 33). Besides of these irreversible PSA complexes, there are non-catalytic PSA forms which don't bind to the inhibitors and are commonly referred to as "free PSA". Free PSA is proteolytically processed and creates its inactive type which is not able to form complexes with the inhibitors. This type of PSA is not implicated in the cleavage and proteolysis of vascular substrates (34, 35). In the same manner, it is hypothesized that most non-catalytic forms of KLK2 do not also appear to bind to the mentioned inhibitors in the blood.

Recently, a new complex of KLK2 with protease inhibitor 6 (PI-6) has been discovered in prostate cancer tissue. PI-6 is an intracellular serine protease inhibitor with both anti-trypsin and anti-chemotrypsin activity. It is estimated that this 64-kDa KLK2-PI6 complex comprises 10% of total KLK2 in prostate tissue, whereas it increases in prostate tumor tissue. The KLK2-PI6 complex provides evidence which may indicate tissue damage and necrosis associated with neoplasia and therefore play a vital role in the development of neoplasia (36). Moreover, KLK2 may be observed as complexes with protein C (PC), ACT, α2-antiplasmin, and antithrombin III in the blood, seminal plasma, and prostate cancer tissue.

One the other hand, KLK2 without complex (inactive) or free form of KLK2 in serum includes the following (37):

- Decayed KLK2
- Denatured KLK2
- Pre-enzyme form of KLK2 or zymogen

Prostate cancer and KLK2 as biomarker

Prostate cancer is the second cause of death in men with the highest number of new cases among all cancers. This type of cancer is highly dependent on the androgen signaling pathway in order to proliferate and survive. Therefore, cancer dependence on androgen is a benefit to treat the disease though androgen deprivation. The process involved in this disease leads to destruction of the basement membrane, destruction of the basal cell layer and glandular structure (38). Prostate cancer is usually diagnosed by digital rectal examination or PSA test in the blood. Numerous studies indicate that an increase in level of PSA in the blood is a sensitive (not specific) way which is associated with the diagnosis of the risk or occurrence of prostate cancer as well as usage as an effective factor in monitoring relapse after treatment (16).

KLK2 is well-known as the second biomarker among all of the kallikrins in detecting prostate cancer. Various histological studies have confirmed an increase in the ratio of KLK2 to PSA expression during carcinogenesis and progression of prostate cancer. Therefore, it was hypothesized that KLK2 may be a useful biomarker for the diagnosis of advanced prostate cancer (2). The KLK2 expression in patients with prostate cancer is higher than the healthy people, therefore, KLK2 gene could be considered as a useful factor in prostate cancer, whose expression is contributed to the

development of prostate cancer (39). Concentrations of PSA and KLK2 in tissue is 10^6 times higher compared to concentrations in blood. Recent research on prostate volume in prostatectomy specimens have shown that serum KLK2 concentration is significantly associated with extracapsular extension (ECE) of prostate cancer (2, 40).

Statistical analysis has revealed that the ratio of KLK2 to free PSA (fPSA) can specifically diagnose prostate cancer from benign prostatic hypertrophy. Therefore, the usage of KLK2 as a crucial diagnostic marker may increase the specificity of prostate cancer diagnosis. The prognostic information can be obtained by measuring KLK2. High level of KLK2 expression along with a low ratio of free PSA to total PSA (tPSA) are associated with prostate cancer malignancy and may also predict disease recurrence after local treatment (2, 41, 42). Therefore, it suggests that assessment KLK2 in patients with prostate cancer is a valuable biomarker which can be used to evaluate the clinical outcome and select the most appropriate therapy strategy.

KLK2-targeted therapies for prostate cancer

Due to the critical role of KLK2 as a prostate cancer biomarker in the diagnosis of this type of cancer, this biomarker has also been introduced as an important therapeutic target for the treatment of prostate cancer. There are several naturally inhibitors such as serpins, Kazal-type serine protease inhibitors and α2-macroglobulin which inhibit KLKs, however they act non-specifically (43). Recently, a KLK2 inhibitor that is a modified version of ACT (MD-PK67b) has been shown to reduce the development of prostate cancer tumors producing KLK2 (44). In addition, prodrugs activated by KLKs have been also described by some studies (45, 46). These prodrugs are capable to deliver drugs to a specific tissue. The inactive form of a prodrug comprises of a toxic molecule conjugated to a peptide. The activated prodrug is generated in the target tissue through cleavage of the peptide by a specific protease, resulting in release of the active form of drug molecule (43). A KLK2-activated prodrug consisting of a KLK2 peptide substrate conjugated to the thapsigargin analog, L12ADT, has shown anti-tumor activity in human prostate tumors (47).

CONCLUSION

KLK2 is a trypsin-like serine protease which is highly expressed in prostate tissue. Beside of the physiological functions of KLK2 to maintain normal cell and tissue integrity and homeostasis, androgen-regulated KLK2 gene expression can also contribute to prostate malignancies and the progression of prostate cancer. Mechanistic roles of KLK2 in the progression of prostate cancer are included the activation of plasminogen activator urokinase, inhibition of uPA inhibitors, activation of MMPs and proteolytic degradation of extracellular matrix proteins. Moreover, KLK2 induces proliferation of cancer cells as well as inhibition of apoptosis through activating IGF factor and MAP kinase signaling pathway, regulating PTHrP protein activity and degrading IGFBP3 protein. KLK2 also plays an important role in promoting migration, metastasis and angiogenesis of prostate cancer cells. Therefore, KLK2 can be recognized as the second diagnostic biomarker of prostate cancer. It is specifically able to distinguish prostate cancer from benign hypertrophy, thus it increases the specificity of prostate cancer diagnosis. In conclusion, evaluation of KLK2 provides a valuable method for future clinical procedures that can be used to assess the clinical outcome and select the most appropriate treatment for patients with prostate cancer.

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Relationship between LncRNAs and Multiple Sclerosis (MS)

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Abstract:

Multiple sclerosis (MS), the most common inflammatory demyelinating illness of the central nervous system (CNS), presents a range of clinical symptoms. The body's immune system attacking myelin causes the transmission block in MS, which increases the electrical capacity of axons. Studies suggest that epigenetic factors play a part in the development of MS. Longer than 200 nucleotides in length and widely distributed, lncRNAs are linear RNA transcripts that cannot code for proteins. For instance, evidence suggests that lncRNAs are essential for a number of cellular functions, including immune response regulation, epithelial mesenchymal transition (EMT), cancer cell proliferation and metastasis, cellular homeostasis, and embryonic development. Epigenetic mechanisms have been proven to have a significant impact on the pathophysiology of MS, and their participation has revealed the function of lncRNAs as epigenetic regulatory molecules in molecular processes. The major subjects of this study have been the relationship between lncRNAs and MS, the role of lncRNA in the pathophysiology of the disease, and the diagnostic and prognostic potential of lncRNA in MS.

INTRODUCTION

Because the motor, sensory, visual, and autonomic systems are affected, multiple sclerosis (MS), a chronic inflammatory illness, manifests as inflammation of the central nervous system (CNS) (1). The main symptoms of MS are optic neuritis, which is an inflammation of the optic nerve; Uhthoff's phenomenon, which is a temporary worsening or fluctuation of MS symptoms accompanied by an increase in body temperature; and Lhermitte's phenomenon, which is an abnormal electric shock to the spine and back of the neck that results in radiation of the arm or leg. It was previously believed that MS is primarily a T-cell-mediated autoimmune illness and that the most well-known mechanisms, such as human leukocyte antigen (HLA) associations, are the result of hereditary variables known to increase the vulnerability of MS patients(2). Clinical classification has not been altered in more than 20 years, despite the expansion of various therapeutic treatments based on disease-modifying medications. On the other hand, there is currently no effective treatment for multiple sclerosis that is in its advanced stages (3). Lack of understanding of the underlying processes causing advanced MS is probably one of the causes. Recent criteria have been developed to characterize the course of the illness because MS has a gradually progressing course and people with MS experience a wide variety of symptoms.Many immunological and non-immune-linked ailments, such as cancer, autoimmune diseases, and infectious diseases, are brought on by impaired immune responses; yet, the underlying process is still poorly understood (4). Long non-coding RNAs (lncRNAs) have recently been demonstrated to have a critical role in regulating the immune response, immune cell growth, and immune system development. But so far, only a small number of lncRNAs have been shown to play a role in controlling the immune system (5). LncRNAs' capacity to regulate gene expression and their part in the pathophysiology of illness have just recently come to light. Despite the fact that studies on lncRNAs and their relationship to MS are still in their infancy, it has been noted that lncRNA-associated disorders in humans result from their abnormal expression. Using their involvement in signaling networks and the control of gene expression as our main points of focus, we review the lncRNAs implicated in the

pathogenesis of MS in this study(6).

Structure LncRNAs

The eukaryotic genome does have an extremely intricate structure. The human genome does not encode proteins in over 98% of it. The Human Genome Project's (HGP) complete identification of the human genome has resulted in the discovery and mapping of new human genes (7). High-throughput sequencing methods like next-generation sequencing (NGS) have revealed a whole new regulatory environment made up of lncRNAs. Currently, more than 28,000 lncRNA genes have been identified (8). Intergenic lncRNAs (transcribed entirely from introns of protein-coding genes), processed lncRNAs, overlapping lncRNAs (which contain an encoding gene in the intronic region), antisense lncRNAs (which are the opposite strand of protein-coding genes and can be both multi-exonic and -intronic), and intronic lncRNAs have all been classified as lncRNAs (9). Similar to how mRNA is processed, the majority of lncRNAs are processed via 5' end capping, splicing, 3′ end cleavage, and polyadenylation (10). LncRNAs have a variety of biological functions in the nucleus and cytoplasm and are polyadenylated and catalyzed by RNA polymerase II. LncRNAs only impose extremely light sequence restrictions and gain secondary and tertiary structures (11). As a result, it is assumed that the majority of lncRNAs have more than two exons. LncRNAs are very prevalent, varied linear RNA transcripts that are longer than 200 nucleotides and do not function in the production of proteins (12). Small open reading frames (sORFs), which are found in a number of lncRNAs but do not encode proteins, have recently been demonstrated to be converted into functional small proteins (13). LncRNAs localized in the nucleus interact with genomic DNA transcription factors, chromatin, spliceosomes, and other nuclear proteins that affect transcriptional and epigenetic regulation (14). They also play a critical role in chromatin organization, transcription, and post-transcriptional modifications. LncRNAs' functions, like those of proteins, are based on where in the cell they are found (15). Numerous lncRNAs display distinctive nuclear localization patterns and appear to be involved in altering nuclear performance. The immune system, tumorgenesis, epithelial-mesenchymal transition, cancer cell proliferation and metastasis, cellular homeostasis, and even embryonic development have all been shown to be impacted by lncRNAs in recent years (16). Numerous studies have demonstrated a strong association between cancer-related genetic polymorphisms and lncRNAs as functional genomic components. The aetiology of autoimmune illnesses may also be influenced by lncRNAs, according to new research, which also implies that they play a significant role in immune system regulation(17).

Various forms of sclerosis and their causes

There are many different clinical signs of MS, which is an inflammatory illness and demyelinating disease in the CNS, particularly in the spinal cord, optic nerves, and brain.Multiple localized regions of myelin degradation inside the CNS are the pathologic characteristic of MS, a chronic inflammatory condition that damages the CNS (18). Thus, the fundamental pathophysiological mechanism causing the conduction block is increasing neurodegeneration brought on by the breakdown of myelin, which is the primary outcome of autoimmune assaults in MS. This neurodegeneration increases the electrical potential of axons (19). Inflammation and blockage of nerve conduction appear to be the most significant variables involved in the pathogenesis of MS, despite the fact that it is a complicated illness with an unresolved underlying mechanism in its pathogenesis and etiology (20). However, MS is at least twice as common in women as it is in men, suggesting that epigenetic pathways play a role in the development of MS (21). Genetic factors do appear to be the most significant components involved in the etiology of MS. Smoke use, sun exposure, the Epstein-Barr virus (EBV), DNA methylation patterns, non-coding RNAs, and epigenetic determinants, including histone modifications, are examples of environmental influences (22,23). Smoking has also been linked to an increased risk of MS impairment progression. Epstein-Barr virus-related infections and MS have been linked, according to serologic and epidemiological research (24). A number of miRNAs, a family of short non-coding RNAs that interact with LncRNAs to control host gene expression, are also encoded by the EBV genome (25). These suggested an EBV and MS connection that could exist. Relapsing-remitting MS is linked to vitamin D deficiency. Low levels of vitamin D lead to immunodeficiency against viral agents because it regulates immune system activity (26). High dosages of vitamin D have been proven to lower interleukin-17 in clinical trials and observational research, but they have no effect on other inflammatory markers (27). The onset and development of MS may be impacted by epigenetic changes such as DNA methylation, histone modifications, and post-transcriptional gene silencing carried out by microRNAs (28). Regardless of the stage of the disease, there were significant changes in the DNA methylation profiles of T helper cells (CD4+ T cells), cytotoxic T cells (CD8+ T cells), and whole-blood acquired from MS patients (29). Evidence shows that, in contrast to the control group, hypermethylation only affects cytotoxic T cells and not helper T cells or genomic DNA taken from the whole blood of MS patients(30). The methylation of CpG sites across the individual's genome did not differ significantly.Genes associated with the immune system are expressed

excessively in MS patients, according to genome-wide association studies (GWAS) (31). Recent genome-wide association studies in MS have identified the genetic factors that contribute to this polygenic disease and more than 100 risk loci associated with the disease (32). However, every single locus, with the exception of the specific HLA-region genes, only marginally increases disease risk (33). Nucleotide polymorphisms work in a certain way to increase the risk of illness in a population, and this is how MS develops. A limited number of signals are linked to splicing alterations; however, the majority of these frequent polymorphisms do not impact the protein sequence of translated products (34). In fact, the majority are located in intronic regions flanking genes. So far, nothing is known about the basic mechanisms underlying MS parthenogenesis, including its pathogenesis (35). As a result, the molecular mechanisms involved in the pathophysiology of MS and their etiology are still poorly understood. The chance of acquiring MS varies from person to person, with Caucasians having a higher risk than Asians and Spanish people (36). LncRNAs may have a role in the development of autoimmune illnesses as they regulate a number of biological activities and immune responses. There have been recent reports linking lncRNA-containing microvesicles to AIDS (37).

Results of the MS and lncRNA association

Studies have shown that lncRNAs have a role in MS progression and control B cells and CD4+ T-helper cell differentiation. The growth-promoting gene known as BDNF (brain-derived neurotrophic factor) is recognized for its critical contribution to neuronal protection (38). The release of BDNF by neurons, T cells, macrophages, astrocytes, and microglia cells in an MS patient was demonstrated to have polytropic effects on immune cells that result in inflammatory reactions (39). It has been discovered that the lncRNA BDNF-AS, also known as BDNF-AS, suppresses the transcription of BDNF in various cells, acting as a negative BDNF regulator. BDNF-AS and BDNF were found to have a significant association in people with MS illnesses (40). The lncRNA known as GAS5 (specific for growth 5) was first discovered in a research study to be involved in the suppression of glucocorticoid receptors (GRs) in MS patients (41). Glucocorticoids might be thought of as a possible therapeutic agent in inflammatory and autoimmune illnesses since they have a significant impact on immune system regulation (42). By attaching to the DNA domains of glucocorticoid receptors, GAS5 can block glucocorticoid-dependent responses (GRs). Gharesouran et al. demonstrated a relationship between GAS5 and NR3C1, the gene that codes for the glucocorticoid receptor (Nuclear Receptor Subfamily 3 Group C Member 1) (43). Additionally, Sun et al. demonstrated in different research that GAS5 interacts with PRC2 (the polycarbonate-2 suppressor complex) and inhibits the IRF4 transcription factor. As a result, it inhibits T-cell growth. Additionally, GAS5 enhances the polarization of the M1 microglia subgroup, which plays a role in MS pathogenesis, while inhibiting the M2 microglia polarization (44). Mammalian target of rapamycin complex 1 (mTORC1) is known to be inhibited by DNA damage-inducible transcript 4 (DDIT4), a cytoplasmic protein that promotes DNA damage in response to cellular stressors. A molecule called mTORC1 is involved in the development and expansion of T lymphocytes (45). According to Zhang et al., lncRNA DDIT4 (lncDDIT4) and DDIT4 were highly expressed in MS patients. The DDIT4/mTOR signaling axis is a target of lncDDIT4, which has a significant impact on Th17 differentiation (46). Mammals have an abundance of MALAT1, often referred to as NEAT2 (nuclear-enriched abundant transcript 2). The long noncoding RNA (lncRNA) MALAT1 (metastatic lung adenocarcinoma copy 1), which is housed in the cell nucleus, controls the transcription and maturation of RNA as well as the expression of many different genes. The neurological system, endocrine organs, the stomach, the bone marrow, and the lungs all express MALAT1 more than other tissues. Masoumi et al (47). discovered that primary activated macrophages and splenocytes express MALAT1 more highly. A shift in the differentiation of macrophages to a pro-inflammatory M1 phenotype, which releases a variety of inflammatory cytokines, has been shown in macrophages treated with specific MALAT1 siRNAs (48). Additionally, by inhibiting Treg differentiation and stimulating T cell differentiation to pathogenic Th1 and Th17 phenotypes, the reduction of MALAT1 expression in CD4+ T cells further increases the proliferative capacity of T cells (49). These results show that MALAT1 is involved in triggering anti-inflammatory responses. Additionally, they discovered that inhibiting MALAT1 increases CD4 T cells' capacity for proliferation, which is associated with a striking increase in the number of Th17 cells that produce IL-17 and IFN-producing Th1 cells while decreasing the number of Foxp3-positive (regulatory T lymphocyte) cells (50). MALAT1 has been shown to have an effect on the AS (alternative splicing) of pre-mRNAs in WI-38 and HeLa cells that control the activity of SR proteins. Its capacity to attach to other splicing elements, such as a number of hnRNPs that affect its own expression, has also been proven (51). The findings of a different investigation showed that MALAT1 regulates the expression of splicing factors as well as MS-related alternative splicing events, which strongly implies that it plays a role in MS pathogenesis (52). Dendritic cells (DC) express

long non-coding RNA (Lnc-DC), which can mediate DC maturation via phosphorylation transducers and transcription activator 3 (STAT3). Through the transcription of downstream genes, Lnc-DC has been demonstrated to have a role in the differentiation of monocytes into DC and the activation of T cells. As a result, LNC-DC can distinguish between young and mature DCs (53). A thin line of evidence suggests that MALAT1 and lnc-DC serum levels may be potentially promising indicators in MS preliminary screening, suggesting that these lncRNAs may be essential in the development of MS illness (54). MALAT1 and lnc-DC have been suggested to be used as treatment strategies in MS, which is encouraging. In a study, it was discovered that three long noncoding RNAs (lncRNAs) called taurine-up-regulated gene 1 (TUG1), nuclear paraspeckle assembly transcript 1 (NEAT1), and P21-associated ncRNA DNA damage activated (PANDA) regulate immune responses and DNA damage responses (DDR) in MS patients (55). NEAT1 expression was found to be inversely related to the age at which the disease began and the length of the illness in female patients (56). TUG1 expression was also inversely correlated with the typical illness duration in female patients.In response to DNA damage, the interaction of TUG1 with p53 and PANDA controls the expression of genes that govern the cell cycle and stabilizes the p53 protein. Additionally, NEAT1 controls the production of cytokine genes, including interleukin (IL)-8, that are implicated in antiviral responses (57). In research by Imamura et al., a NEAT1-dependent SFPQ (Splicing Factor Proline and Glutamine Rich) translocation was shown to suppress IL-8 transcription, activate NEAT1 expression, move SFPQ from the IL8 promoter to the paraspeckles, and finally result in the transcriptional activation of IL8 (58). NEAT1 is important for innate immune responses because it controls the transcription of antiviral genes through SFPQ and NEAT1's stimulus responsiveness (59). LncRNAs are drawing increasing attention to the function of antisense noncoding RNA in the INK4 locus (ANRIL), which controls cell proliferation and senescence. ANRIL's regulatory function in inflammatory responses has led to increased interest in its potential significance in inflammatory diseases (60). According to research findings, ANRIL has a role in the etiology of MS. LincMAF-4, by regulating Th1/Th2 differentiation, has been considered one of the main drivers in MS pathogenesis, despite the fact that MS is an autoimmune disease that is related to immune dysregulation and an imbalance in Th1, Th2, and Th17 cells (61). However, more research is required to confirm this. In a newly released study, it was shown that linc-MAF-4 was markedly up-regulated in MS patients, indicating that it might control the development of Th1/2 cells (62). Linc-MAF-4 has been presented as a newly discovered member of the lncRNA family that plays a role in the pathogenesis of MS. It has also been demonstrated that the antisense lncRNA FAS antisense transcript 1 (FAS-AS1) regulates the activity of the soluble Fas receptor (sFas) (63). This lncRNA modifies the Fas:sFas ratio and prevents exon skipping during the transcription of the Fas mRNA, leading to the development of Fas ligand (FasL)-mediated apoptosis. It does this by binding to the RNA-5 binding protein (RBM5) (64). It has been documented that this pathway affects lymphocyte growth and immunological responses by modulating apoptosis. It has been emphasized how important TNF and heterogeneous nuclear ribonucleoprotein L (THRIL) are as lncRNAs that are linked to innate immunity (65). After the innate activation of THP1 macrophages, it was chosen among a vast number of differentially expressed LncRNAs. Additionally, Eftekharian et al. demonstrated dysregulation of three lncRNAs, including FAS-AS1, THRIL, and plasmacytoma variant translocation 1 (PVT1), in MS patients. OIP5-AS1 was identified for the first time as a critical factor in early CNS development in zebrafish. It has been demonstrated that OIP5-AS1 decreases the cyclin G-associated kinase (GAK) mRNA stability required for mitotic development (66). These findings imply that OIP5-AS1 inhibits cell growth via lowering GAK levels in combination with RNA-binding proteins like HuR1. HuR1 accessibility seems to be restricted to the cyclin D1, cyclin A, and SIRT1 (silent information regulator 1) target mRNAs. Additionally, the results demonstrated that aberrant mitosis followed the down-regulation of OIP5-AS1 and was caused by a rapid up-regulation of GAK regulation, suggesting that OIP5-AS1 was at least repressed by lowering GAK expression. HUR1, a protein that interacts with OIP5-AS1 conserved sequence motifs, appears to have an effect downstream of OIP. AS1 has been found to be expressed in inflammatory diseases such as MS and encephalomyelitis, astrocytes, and the HUR1 gene (67).

CONCLUSION

The relevance of lncRNAs in the pathogenesis of MS has been shown through altering epigenetic mechanisms and their function in molecular processes. Epigenetic mechanisms have been found to play a significant part in the development of MS. Additionally, it has been shown that the abnormal expression of several lncRNAs is closely associated with the development of various tumors, leading to the consideration of many lncRNAs as possible therapeutic targets, stand-alone prognostic predictors, and important biological markers in malignancies. Several studies have indicated that LncRNAs are important direct targets for therapeutic treatments in hepatic illnesses. Due to their functional

role in controlling the expression of numerous genes at the transcriptional level or after transcription, along with proteins and signaling pathways, these studies identify LncRNAs as a key factor in the pathogenesis of MS. This information may one day be used to develop a therapeutic strategy for MS patients. But research in this area is still in its early stages, and more work needs to be done to figure out what LncRNAs do in MS.

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Vitamin D Binding Protein Gene Polymorphisms and its Association with Type 2 Diabetes Mellitus in an Iranian Population

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Abstract:

Polymorphisms of Vitamin D-binding protein (DBP) may represent a risk factor for susceptibility to Type 2 Diabetes Mellitus (T2DM). Two polymorphisms are common at codons 420 (ACG to AAG) and 416 (GAT to GAG) in the DBP gene. The present study aimed to assess variants of DBP at codons 420 and 416 utilizing polymerase chain reaction-restriction fragment length polymorphism(PCR-RFLP), which were digested by Sty I (codon 420) and Hae III (codon 416) restriction enzyme. For this purpose, 240 patients were recruited along with 159 controls. The genotype frequency of Glu/Glu at codon 416 in control and patient groups was 25.16% and 32.92% respectively. Moreover, the genotype frequency of Lys/Lys was 6.67% in patients and 1.89% in controls at codon 420. A significant difference was found between control and patient groups in genotype frequencies at codon 420 ($p<0.05$). It was found that there might be an association between the vitamin D-binding protein gene and T2DM.

INTRODUCTION

Diabetes is among the most common metabolic disorders, for which the number of suffering patients is estimated as 200 million people all over the world. There are two main classifications for Diabetes: type 1 and type 2 (1 ,2). Type 2 diabetes mellitus is non-insulin-based and the main cause of 90% of the extensive disease (3). It can be affected by interactions between the environment and genes (4). The usually identified genes are T2DM CAPN10, KCNJ11, PPARG, ABCC8, HNF4A, HNF1A, GCK, PC-1/ENPPI, PTPN1, IRS, and LMNA (5). Vitamin D has a key role in the control of bone metabolism and mineral homeostasis. In addition to its main roles in physiological development, the endocrine system of vitamin D contributes to several pathological progressions like autoimmune disease, coronary artery disease, and T2DM (6,7) . Recently, a huge deal of attention has been paid to the correlation between T2DM and vitamin D disorders (8,9). According to uncertain available data in this regard, the vitamin D pathway affects the evolution of diabetes. 1,25-dihydroxy vitamin D3 can bind to β-cells thus affecting insulin release (10). Experimentally, it was revealed that vitamin D deficiency will reduce the secretion of insulin (11). There is a relation between insulin disorder and the reduced levels of DBP (12). According to a survey, insulin secretion can be improved by vitamin D supplements (13). There is also an association between vitamin D levels and genes of DBP, VDR, CYP24A1, CYP2R1, CYP27A1, and CYP27B1(14-17). DBP is critical for vitamin

D metabolism and affects the levels of vitamin D and insulin release in β-cells (18,19). Vitamin D binding protein also known as group-specific component protein (GC), is a multifunctional serum glycoprotein. Considerably, DBP is a series of the albumin, α-albumin/afamin gene, and α-fetoprotein family. DBP has a molecular weight of almost 52-59 kDa. It has been synthesized as a glycoprotein in the liver (20,21). The human DBP gene localizing on 4q12-q13 includes 12 introns and 13 exons. DBP gene is highly polymorphic with 3 common variants (GC1S, GC1F, and GC2) and more than 124 scarce variants (22). These three usual variants are resultant of sequence variations at codons 420 and 416 in exon 11 of the DBP gene. At codon 416, aspartic acid is replaced by glutamic acid via a nucleotide substitution from GAT to GAG. Moreover, threonine is replaced by lysine through a nucleotide substitution of ACG to AAG at codon 420 (23). In the southern coastal part of Iran (Bandaries populations), there is a considerably higher prevalence of diabetes than its mean prevalence in the country (12% and 7.7%, respectively). Furthermore, the Bandaries' inhabitants seem to be negroid and different ethnically from the northern and central white population of Iran. In this study we sought to explore the frequency of these SNPs in T2DM patients.

Methods

Collection of samples

This study was performed on 240 T2DM patients and 159 non-diabetics as a control group (age range of 30–70 years for both groups). All the participants were natives of southern Iran (Bushehr Province) referring to the Persian Gulf Tropical Medicine Research Center and Fatemeh Zahra University Hospital in Iran. The medical-ethical committee of Bushehr University of Medical Sciences verified the study and each participant confirmed the informed consent. Data collection was performed through anthropometric measurement and a questionnaire. WHO criteria and definitions were utilized for non-diabetic and diabetic cases (Fasting Plasma Glucose ^{3}7.0 mmol/l or 126mg/dl, as well as 2-h post-load plasma glucose ^{3}11.1 mmol/l or 200mg/dl during a 75-g Oral Glucose Tolerance Test).

DNA extraction and polymorphism determination

The blood samples were collected from participants who were in the night fasting condition. Genomic DNA of blood samples was extracted utilizing QIA amp DNA Mini Kit (Germany), based on the "blood and body fluid spin protocol" and the manufacturer's recommendation. PCR-RFLP was used to examine polymorphisms at codons 420 (ACG to AAG substitution) and 416 (GAT to GAG substitution) in exon 11 of the DBP gene. Gene Runner software was used to design forward and reverse primers for the DBP gene followed by acquiring gene sequences from Gene Bank (http://www.ncbi.nlm.nih.gov). Table 1 presents the oligonucleotide primers utilized in this work. PCR amplification was conducted in a 15μL reaction mixture containing 0.4 mM of dNTP, 1.5 μL 10 X PCR buffer, 20 pmol of each forward and reverse primers, 0.5 units of Taq polymerase, 40 ng of DNA template, and 1.6 mM MgCl2 via PCR thermocycler model (Techne TC-512). The PCR conditions were as follows: the first denaturation cycle of DNA for 5 min at 95°C after 35 cycles, each containing 35 s of denaturation at 95°C,and 20 s annealing at 52°C, and 30s extension at 72°C, and the ultimate extension cycle of 72°C for 10 min.PCR products were 483bp (base pairs), and each PCR product (10μL) was separately digested with 1μL of the HaeIII and StyI restriction enzyme for 1 h at 37°C. Table 1 represents the results of PCR-RFLP analyzed on ethidium bromide-stained 3% agarose gel. Linkage disequilibrium was determined between codons 420 and 416 in the DBP gene by Haploview software. The chi-square goodness-of-fit test was used to assess Hardy-Weinberg Law. A p-value less than 0.05 is particularly regarded to be statistically significant.

Table 1. PCR-RFLP and primer sequences for DBP gene

Gene	Primer sequences 5′-3′	PCR product size (bp)	Detection methods
DBP	F -ACTAGTAGTAAGACCTTA R-GATTGGAGTGCATACGTT	Normal allele: 483 Mutant allele: 186, 298	**PCR-RFLP** **HaeIII**
		Normal allele: 483 Mutant allele: 178, 305	**PCR-RFLP** **StyI**

RESULTS

Table 2 presents clinical characteristics revealing no difference between control and patient groups. Nevertheless, patients had higher levels of insulin than control groups and there was a significant difference between aforementioned groups (P=0.02). Hardy-Weinberg equilibrium was the basis for the genotype and allele frequencies in control and case at codons 416 and 420. Moreover, there was linkage disequilibrium for the mentioned codons in both groups (Table 3). No significant differences were found between control and patient groups in the frequency of haplotypes in the DBP gene at codons 416 and 420 (Table 4). Moreover, the odds ratio for haplotypes is determined (Table 5). It should be noted that no significant association was found in allele frequencies of the study population (Table 6). A considerable association was revealed between patient and control groups by the genotype frequencies of Thr –Thr/ Thr – Lys/ Lys- Lys in the DBP gene at codon 420 (P<0.05). However, no significant relationship was found at codon 416 in genotype frequencies (Table 7). For Glu/Glu (codon 416) and Lys/Lys (codon 420), the genotype frequency of patients was higher significantly than controls.

DISCUSSION

DBP is essential for the role of vitamin D in the cell. There are no considerable studies in the field of the relationship between T2DM and vitamin D. However, evidence suggested that vitamin D can enhance the sensitivity to insulin and the survival of pancreatic cells (24) . Some studies have reported a relation between lower rates of vitamin D in serum and increasing T2DM(25). In this work, the relation of T2DM with the DBP gene was studied in Bushehr province. Gcls, Gc1f, and Gc2 can be referred to as prevalent variants in the DBP gene(22). These three variants are in the codons 420 and 416 in exon 11 in the DBP gene(23).

Table 2. The clinical characteristics of the groups of patients and controls

Features	Patients (n=240)	Controls (n=159)	p-value
Female/male ratio	124/116	76/83	0.4
Age (years)	41.02 ± 9.52	41.59 ± 9.84	0.6
BMI (kg/m2)	26.96 ± 4.61	26.13 ± 4.52	0.08
Fasting glucose (mg/dl)	81.2 ± 9.24	81.84 ± 10.37	0.5
2 h-OGTT (mg/dl)	92.47 ± 17.18	92.29 ± 17.49	0.9
Insulin (µIU/ml)	8.24 (5.84–15.07)	6.97 (5.83–11.12)	0.02

Table 3. Analysis of linkage disequilibrium between 420 and 416 codon

	|D'|	r2	LOD	LOD_p_value	Chi_square	p_value
control	0.92674	0.35657	64.56365	0	113.3897	0
case	0.58411	0.12608	32.37624	0	60.51963	0
total	0.73278	0.20845	84.47336	0	166.3392	0

Table 4. Haplotype frequencies in DBP gene at codons 416 and 420

Haplotype	T2DM patients	Controls
Asp–Thr	78 (27%)	32 (21%)
Asp–Lys	52 (17.7%)	40 (26.2%)
Glu–Thr	146 (50.1%)	79 (52%)
Glu–Lys	15 (5.2%)	2 (0.8%)
	P-Value=0.3	

Table 5. The odds ratio in haplotypes of the DBP gene

		of OR	of OR
Glu/Thr	0.979	0.737	1.3
Asp/Lys	1.301	0.926	1.828
Asp/Thr	0.661	0.471	0.926
Glu/Lys	6.156	1.418	26.717

Table 6. The allele frequencies of the DBP gene at codons 416 and 420 in the study population

Allele		
Codon 416		
	ASP	**Glu**
T2DM patients	214(44.58%)	266(55.42%)
Controls	150(47.17%)	168(52.83%)
P-Value=0.5		
Codon 420		
	Thr	**Lys**
T2DM patients	370(77.08%)	110(22.92%)
Controls	232(72.96%)	86(27.04%)
P-Value=0.2		

Table 7. The genotype frequencies of the DBP gene at codons 416 and 420 in the study population

Genotype			
Codon 416			
	Asp/Asp	**Asp/Glu**	**Glu/Glu**
T2DM patients	53(22.08%)	108(45%)	79(32.92%)
Controls	31(19.5%)	88(55.34%)	40(25.16%)
P- Value=0.1			
Codon 420			
	Thr/Thr	**Thr/Lys**	**Lys/Lys**
T2DM patients	146(60.83%)	78(32.5%)	16(6.67%)
Controls	76(47.8%)	80(50.31%)	3(1.89%)
P<0.05			

Some studies have been performed on the relationship between this gene with T2DM.

Hirai et al. in a study on the Japanese population found that the DBP gene is effective in getting susceptible to T2DM (26). Moreover, the relation between the DBP variant and type II diabetes was reported by Wang et al (27). in The Chinese population in Shanghai and Shao et al (28). in China, Najing province. It is revealed the effects of DBP genotype on the fasting insulin level in Dogrib Indians. Baier et al (23). represented the relation between the DBP variants to oral glucose tolerance in nondiabetic Pima Indians. However, the relation of T2DM to the DBP gene was not proved by Malecki et al(29). in the Polish population, Ye et al(30). in French Caucasians and Klupa et al(18). in White Americans of European Origin. DBP variants in the Asian population were along with T2DM significantly (31). In the present work, we found no meaningful difference between the patient and healthy population on the frequency of haplotypes and alleles in codons 420 and 416. Nevertheless, we found no significant differences regarding genotype frequency in codon 416. Considering genotype frequency in codon 420, a significant difference was identified in our population studied. Wang et al(31). reported a significant relation between T2DM codons 416 and 420 in DBP variants in the Asian population. They also revealed that people with the Lys allele were highly affected by diabetes in the Asian population. More interestingly, in most studies, the frequency of the Glu-Lys haplotype was close to zero. The frequency of this haplotype in control and patient groups were reported near 0% by Malecki et al(29). and Ye et al. (30). Moreover, this haplotype frequency was reported as 7% and 0% in White Americans of European Origin population in patient and control groups, respectively [18]. Rahman et al(32). declared the lowest level of vitamin D for type 2 diabetic patients and also the highest frequency of Glu/Glu (codon 416) and Lys/Lys (codon 420) (32). Nevertheless, the rate of Glu-Lys haplotype frequency was 5.2% and 0,8% in the patient and healthy groups respectively in our study. Based on the genotype frequency, in codon 420 there was a significant difference between patient and healthy population in our study. It should be noted that diabetes is a disease caused by the interaction of genes, lifestyle, and environment (33). Generally, more studies are required with different populations to obtain a reliable conclusion on the relationship between gene DBP variants and T2DM.

Conflict of interest

There are no conflicts of interest.

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Personalized Medicine Approach and the Application of iPSCs in Neurological Diseases

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Abstract:
A number of animal disease models have been created in the past to investigate the molecular basis of neurological diseases and identify novel treatments, but their effectiveness has been limited by the absence of comparable animal models. There are still several important problems that need to be overcome, including the high expenses associated with creating animal models, ethical issues, and a lack of similarity to human disease. More than 90% of medications fail in the last stage of the human clinical trial as a result of inadequate early screening and assessment of the molecules. A novel strategy based on induced pluripotent stem cells has been developed to get around these restrictions (iPSCs). A new road map for clinical translational research and regeneration treatment has been made possible by the discovery of iPSCs. In this paper, we investigate the potential use of patient-derived iPSCs to neurological disorders as well as their significance in scientific and clinical studies for the creation of disease models and a road map for the next of medicine. The role of human iPSCs in the most prevalent neurodegenerative illnesses (such as Parkinson's and Alzheimer's disease, diabetic neuropathy) was evaluated. The patient-on-a-chip idea, where iPSCs may be cultivated on 3D matrices within microfluidic devices to produce an in vitro disease model for tailored medication, is another new development in the field of personalized medicine that we looked into.

INTRODUCTION

The development of in vitro disease models for a variety of ailments, including neurodegenerative disease, diabetes mellitus, and heart, liver, lung, and kidney disease, was made possible by the discovery of induced pluripotent stem cells (iPSCs) technology in 2007 (1). It is crucial to develop a more suitable drug discovery strategy to close the gap between pre-clinical research and human clinical trials. Reprogramming differentiated cell types into pluripotent stem cells, such as patient fibroblasts or peripheral blood mononuclear cells (PBMCs), has been created and employed for drug testing, greatly enhancing the disease model system for in vitro drug research. This technology is being used to research neurological diseases such as ataxia, amyotrophic lateral sclerosis, multiple sclerosis, spinal cord injury and Parkinson's disease (2). Additionally, it makes it possible for researchers to study and comprehend how complex human tissues, including the brain and heart, respond to newly found medications. In this strategy, blood cells or biopsies are used to create and maintain patient-specific iPSC cell lines (3). The illness state is replicated in vitro in a petri dish using these iPSCs that have been reprogrammed into certain cell types of interest. Because of their propensity to proliferate and differentiate, human-iPSCs can be used to study the physiology of impacted cell types on tissue culture plates. This approach may also be used to test and find disease-specific medicines in a petri dish in vitro. These pre-clinical investigations in petri dishes offered the first proof of concept and a viable method for studying disease molecular mechanisms and screening promising compounds for medication development and cytotoxicity research (4).

Surprisingly, only medications that have been evaluated and pre-tested in pre-clinical research are being examined in human clinical trials. Due to the severe assessment requirements in pre-clinical research, these applicants should ideally work in human clinical trials. However, a significant disparity has been seen between human clinical trials and pre-clinical studies. For example, despite large financing prospects for clinical trials (up to USD 42.5 billion), the outcomes in Alzheimer's disease have been dismal, with a 95% failure rate. Furthermore, only six medications suggested for Alzheimer's disease (AD)

were licensed by the US Food and Drug Administration (FDA) between 1995 and 2021 (5).

We examine the role of human iPSCs in scientific and clinical research in this review. We also look at recent iPSC-related breakthroughs in clinical research and examine the importance of iPSCs in cellular treatment, personalized medicine, and ongoing clinical trials for Parkinson's disease (PD) and Alzheimer's disease (AD).

What are iPSCs ?

Yamanaka, S., and Takahashi, K., made a significant discovery in the early 2000s when they successfully produced new types of stem cells, known as induced pluripotent stem cells, from mouse embryonic and adult fibroblast cultures (iPSCs)(6). OCT4, SOX2, KLF4, and C-MYC, collectively known as the Yamanaka factors, were added to the culture media in order to change how the fibroblasts' genome was expressed. The Yamanaka factors work with viral vectors, particularly retroviral and lentiviral transduction, to help induce and maintain the pluripotent state. iPSCs have been extensively employed for research on possible cell therapies, disease modeling, and pharmacological screening of neuroprotective substances (7).

Additionally, iPSCs with organoids and gene editing techniques like CRISPR-Cas9 transform these cells into a highly adaptable tool for regenerative medicine and drug screening to assess substances with the potential to treat a variety of diseases, leading to the identification of clinical candidates and the approval of some for their application. It's important to recognize their drawbacks, though, including their high cost, the length of time required for development, the requirement to downregulate the MHC (Mayor Histocompatibility Complex) in the host cells if the iPSCs-derived cells will be transplanted in order to limit immune recognition, the unique culture conditions, reprogramming and differentiation processes' contribution to karyotype abnormalities caused by genetic instability, which iPSCs suffer from (9, 10).

Non-integrating techniques, including as synthetic mRNAs, Sendai virus, and episomal DNAs, have been developed in recent years to address the problems caused by genomic instability and lower the hazards associated with vectors (11). Furthermore, genome editing methods offer the chance to incorporate genetic modifications into iPSCs in a site-specific way, creating isogenic iPSCs lines, which are crucial in sporadic and polygenic illnesses (11).

Although technically iPSCs may be produced from any tissue in the body, the most commonly used sources are fibroblasts and peripheral blood mononuclear cells due to their accessibility. The creation of iPSCs begins with the recovery of somatic cells from a patient or an animal model, which are then transduced with a virus comprising the reprogramming factors (12, 13). Once the reprogramming is complete, variations can be seen in the cultured cells, forming colonies that resemble ESCs. As a result, the cells are then gathered for more expansion for several passages to ensure the conservation of their distinctive morphology. To ensure their resemblance, these cells should express ESC antigens such SSEA-4 and TRA-1-80. Other procedures that might be carried out include chromosomal analysis to check for a normal karyotype or to find potential translocations (14).

The function of iPSCs in neurodegenerative disorders

Recent advancements in the capacity to convert patient somatic cells into inducible pluripotent stem cells (iPSCs) have enabled a fresh method of producing disease-relevant cells for in vitro disease modeling (15). Clinicians are in need of novel treatments for neurodegenerative illnesses. Despite sound clinical research, it has been accepted that the medication treatments that have been developed fall short of expectations. Human iPSCs can, in theory, differentiate into any cell type in the human body; thus, patient iPSCs can provide a source of cells with a specific constellation of genetic variants associated with pathogenesis in the appropriate microenvironment (15). As a result, iPSCs are frequently used in well-established human disease models, including both developmental and adult-onset diseases, as two-dimensional (2D) cell cultures or three-dimensional (3D) organoids. Prior to the development of iPSC technology and iPSC-based modeling systems, animal models, primary brain cells, and immortal cell lines all made significant contributions to our knowledge of neurological illnesses. However, all of these models have limits, which drives the urge to create a better modeling system (16). For animal models, species differences constitute a barrier to completely reproduce disease characteristics, resulting in a high failure rate

Table 1. Advantages and limitations of iPSCs technology (8)

Advantages	Limitations
ESC use-related ethical and religious concerns are eliminated.	Reprogramming's efficiency is often low.
Immune rejection is less likely.	Tumorigenesis
Donor cells are quickly and painlessly collected, and no embryos are destroyed.	Insertional mutagenesis risk associated with virus-based delivery methods
Accessible to many patients, as opposed to ESCs which are constrained by ethical consideration.	Increase chance of development of disease due to factors used.

in animal-model-based medication development. In the final stage of the human clinical trial, more than 90% of pre-clinically successful medicines are found to be ineffective (17, 18).

This indicates that human biology is frequently poorly predicted by animal models. Developing a degenerative disease model is a difficult task. Researchers have either altered the expression of a specific gene to create a cell culture model or created a knockout animal model. These models, however, might not be called a perfect illness model that reflects human pathophysiology. It is also extremely difficult to collect human brains postmortem for scientific research due to ethical concerns. Even if a human brain is available, brain tissues for study are extremely degradable and immunologically mature (19). Given its benefits over the previously described modeling methods, iPSC-based disease modeling is becoming increasingly popular for investigating neurological illnesses.Because iPSCs reprogrammed from human somatic cells are derived from humans, they eliminate the issues associated with employing animal models. iPSCs are easily grown and provide an infinite resource for further differentiation into cell types of interest (19). Above all, iPSCs derived from patient somatic cells preserve their original genomic characteristics, such as gene mutations and chromosomal abnormalities (19).

iPSCs in PD

The second most prevalent neurodegenerative condition is Parkinson's disease PD, which is characterized by stiffness, bradykinesia, postural instability, and static tremors. The brains of PD patients experience widespread neuronal loss, particularly due to the steadily deteriorating dopaminergic neurons in the substantia nigra compacta (20, 21). The central and peripheral nervous systems' remaining neurons have inclusion bodies (Lewis bodies) containing -synuclein. Synuclein alpha (SNCA), leucine-rich repeat kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1), parkin RBR E3 ubiquitin protein ligase (PARK2), and cytoplasmic protein sorting (VPS35) are examples of common PD-related mutant genes (21, 22). Additionally linked to PD is the CHCHD2 mutation. The study of PD molecular mechanism has considerably benefited from the development of induced pluripotent stem cells. From PD patients, a successful human iPSC line was created. In order to create iPSCs, Wang et al. (2018) transfected dermal fibroblasts from 52-year-old PD patients with episomal plasmids expressing OCT3/4, SOX2, KLF4, LIN28, and L-MYC. A CHCHD2 mutation is present in the produced iPSCs line (ZZUi007-A) (23). An iPSCs line (201B7) was created by Takahashi et al. in 2007 using the dermal fibroblasts of a healthy donor. At Kyoto University, fibroblasts were reprogrammed utilizing retroviral transduction to express OCT4, SOX2, KLF4, and MYC. They function as a "normal" check (24).

A human iPSCs line (B7PA21) was produced from PD patients with PARK2 mutations by Suda et al. in 2018. Ghrelin receptor expression was shown to be down-regulated in PD-iPSC-derived dopaminergic neurons compared to healthy controls. Additionally, creating the PARK2-KIKO line from 201B7 using CRISPR-Cas9 technology also mirrored the PARK2 gene's lack of function (25). Phospho-ubiquitin signaling was shown to be impacted in human dopaminergic neurons with Parkin or PINK1 mutations by Shiba-Fukushima et al. in 2017. Additionally, it was discovered that human dopaminergic neurons with Parkin or PINK1 mutations have poor control of axonal mitochondrial transport and phospho-ubiquitin signaling (25). Schweitzer et al 2020.'s study demonstrated the autologous transplantation of iPSC dopaminergic progenitor cells into the midbrain of PD patients. Clinical grade iPSCs were created in vitro, evaluated for immunogenicity using a humanized mouse model, and then transplanted into the putamen of PD patients without the need of immunosuppressive drugs. Fluorine-18-L-dihydroxyphenylalanine was used in positron emission tomography to predict graft survival (26). By reprograming OCT3/4, SOX2, c-MYC, KLF4, and BCL-XL, Chen et al., 2021 showed that an iPSCs line could be created from PBMCs of a 32-year-old PD patient with homozygous mutation of c.189dupA in PARK7 (FJMUUHi001). The generated iPSCs were able to display pluripotency markers and differentiate into three germ layers (27).

iPSCs in AD

A neurological, life-limiting, and deadly condition known as dementia causes gradual cognitive decline, behavioral issues, and loss of everyday functioning. The most frequent cause of dementia, accounting for 50% to 70% of dementia cases globally, is Alzheimer's disease (AD) (28). There are 50 million individuals living with dementia globally, according to the 2018 World Alzheimer's Disease Report. An additional incidence of AD is reported every three seconds worldwide, and by 2050, there will likely be 152 million cases (29). It is believed that iPSCs can develop into many different types of cells, including neurons and neurospheres. iPSCs may be utilized to create and automate neuronal subtypes, as demonstrated by experiments done both in vitro and after transplanting cells into the mouse brain (30). For instance, it is possible to analyze the inflammatory response of AD using glial cells produced from iPSCs. iPSCs were employed in another investigation with a mouse model of AD to produce macrophages that could express the A-degrading protease neprilysin (31, 32).

Michael Peitz et al. (2018) showed that using Sendai

virus vectors that encode for the transcription factors OCT4, SOX2, KLF4, and c-MYC, peripheral blood cells from a male AD patient may be transformed into a human iPSCs line. In addition to expressing differentiation into all three germ layers and maintaining the APOE"4/"4 genotype, a significant risk factor for sporadic late-onset AD, the described iPSCs line (33). The iPSCs were created by Liu et al., 2020 from individuals with sporadic Alzheimer's disease (sAD). The Sendai virus, which expresses Oct3/4, Sox2, c-Myc, and Klf4 transcription factors, was used to reprogramme PBMCs (34). An 87-year-old female donor with the APOE3 ("3/"3) alleles' peripheral blood mononuclear cells were used to create iPSCs by Zhang et al. in 2021. The ability of iPSCs to produce pluripotency markers such NANOG, OCT4, and SSEA4 coupled with a normal karyotype was greater than 97% (35). One of the most useful resources for understanding sAD pathogenesis in vitro is the created iPSCs line. Arber et al. demonstrated that iPSCs provide a useful model for examining possible cell dysfunction brought on by genetic fAD mutations by simulating APP processing and A synthesis in the setting of fAD-APP and PSEN1 mutations (36). However, there are significant barriers to the therapeutic usage of iPSCs due to the following unanswered questions: Teratoma development, long-term effectiveness and safety, tumorigenicity, immunogenicity, patient genetic abnormalities, ideal reprogramming, and other factors (37).

iPSCs in diabetic neuropathy

A major cause of death globally, diabetic mellitus (DM) is regarded as a chronic, systemic metabolic disorder. The World Health Organization estimates that there will be 693 million people worldwide with diabetes in 2045, up from just 451 million in 2017 (37). One of the severe microvascular consequences of diabetes mellitus (DM), diabetic nephropathy (DN), is a major risk factor for renal failure in individuals with end-stage renal disease. Although hyperglycemia is a significant risk factor for developing DN, other traits, including glycation end products and the overexpression of certain growth factors, are also connected to its etiology [38]. Additionally, excessive amounts of reactive oxygen might cause the kidney to produce inflammatory cytokines, which quickens the development of DN (38). Unfortunately, there are currently no medications that can stop DN from progressing. The present treatment options are restricted to RAAS blockage, rigorous management of hyperglycemia and blood pressure (39).

DN is linked to negative alterations in the peripheral nervous system, such as myelin degradation and a reduction in nerve conduction velocity (40). The myelin sheath is a multilayered membrane created in the peripheral nervous system by the development of Schwann cells' plasmatic membrane. When peripheral axons are working properly, Schwann cells are crucial because they protect and support both myelinated and non-myelinated peripheral nerve fibers (41). This support consists of both chemical and physical processes, and the release of various neurotrophic chemicals by Schwann cells. Based on this knowledge, many research teams examined the impact of applying Schwann cells to animal models with peripheral neuropathy (41).

Himeno et al. showed that certain mesenchymal stem cells (MSC)-like cells produced from iPSCs engraft to the peripheral nerve and express S100, a Schwann cell marker, when implanted to diabetic mice's thighs. This finding suggests that transplanted cells can actually create peripheral nerve tissue. Additionally, blood flow and capillary density in the soleus muscle of diabetic mice improved after transplantation of MSC-like cells. Therapy with MCS-like cells improved diabetic physiological deficits from a functional standpoint, highlighting the positive impact of such treatment on diabetic peripheral neuropathy (42).

iPSCs have effectively been differentiated into kidney cells in several studies, which may have an impact on how DN is treated. The iPSCs' ability to develop into kidney cells with podocyte features was initially described by Bi et al. iPSCs podocytes enhanced the mRNA expression and protein localization of podocyte markers such synaptopodin, renin, and WT-1 after 10 days of focused differentiation while downregulating the stem cell marker OCT3/4 (43). Human ES cells and iPSCs (referred to collectively as hPSCs) were shown to stably and quickly differentiate into pluripotent cells by chemical induction of the efficient small molecule inhibitor CHIR99021 (CHIR) of GSK-3, which can replicate the formation of mesoderm in developing embryos followed by fibroblast growth factor-2 (FGF-2) and RA and then form tubular structures upon growth factor w. Lam, A. Q. et al (43).

Experimental investigations have shown that MSCs can be employed to treat DN. However, the precise causes of DN have not been fully understood, and the molecular mechanisms for MSC-based DN treatment are currently being researched (44). MSCs' plasticity in regenerative applications was first celebrated since they are multipotent cells with the ectopic capability of homing and differentiating into numerous cell types in response to particular stimuli, including glomerular endothelial cells (44). As far as we are aware, there hasn't been a clinical trial employing iPSCs as a treatment for diabetic neuropathy patients. But there is a fascinating research that employed iPSCs from a patient with idiopathic small fiber neuropathy to identify the best course of action for that patient. The utility of iPSCs in drug development and testing in

general, as well as in customized medicine to find the best cure for diabetic neuropathy or other disorders, is shown by this example.

iPSCs' and personalized medicine

It has been shown that iPSCs play a crucial role in cellular therapy, which might lead to human clinical trials and offer a treatment roadmap in the future (45). Patient-derived iPSCs can also serve as a special model for comprehending how diseases arise. Additionally, it can aid in drug testing and offer fresh perspectives for creating "new future medicines." A brand-new area of personalized medicine based on cellular treatment has emerged: regeneration therapy. A particular drug is created for a patient through personalized medicine using their pharmacogenomic and pharmacogenetic data (45).

The idea of using iPSCs to simulate a disease in vitro is based on their exceptional ability to perpetually divide themselves and their propensity to give birth to every type of cell in the human body (46). iPSCs might therefore offer an infinite pool of cell types that would otherwise not be able to get, such as the motor and dopaminergic neurons afflicted in ALS and PD. The primary benefit of iPSC technology is that it makes it possible to create pluripotent cells from any person in the context of that person's unique genetic identity, including people with sporadic disease and those suffering from complex multifactorial diseases with unknown genetic identities, like autism spectrum disorders (47). A number of recent studies have documented the effective creation of patient-specific iPSC lines from patients suffering from a variety of disorders. However, in a few cases, effective disease modeling has been established. Ebert et al., for example, reported the development of iPSC-derived motor neurons from a patient with a hereditary type of spinal muscular atrophy (SMA), a neurodegenerative illness characterized by the loss of lower motor neurons (48).

Because everyone reacts differently to different types of diseases, it is critical to study personalized medicine or personalized pharmacology. It might be caused by a combination of variables such as genetics, epigenetics, environment, or demographics such as age, gender, and ethnicity (49). These factors, when combined, can accelerate the progression of any disease. However, some authors contend that genetic factors are the most important risk factors in complex diseases, such as neurological disorders. Furthermore, the interaction of genetic, environmental, demographic, and lifestyle factors is critical in disease development (50).

The development of iPSCs technology in 2007 changed the area of personalized medicine by enabling various methods of drug screening; it is also a suitable candidate for tailored cell treatments. The compound attrition rate has a significant impact on drug development costs. Preclinical testing of 5,000-10,000 compounds has been conducted for each medicine that enters the market. More accurate predicted toxicity models would assist in lowering these expenditures (47, 51).

iPSCs also provide interesting potential for high throughput drug screening of particular disease characteristics. This remarkable capacity in toxicity investigations has the potential to improve the efficiency of innovative human medication development while lowering drug attrition in the last phases of development and hence costs (52). The accurate prediction of human drug toxicity is a critical step in the drug development process. Hepatotoxicity and cardiotoxicity, in particular, are two main reasons of medication failure during preclinical testing, while individual response variability to prospective therapeutic agents is also a big issue in effective drug development (52). The benefit of iPSC technology is that it allows for the creation of a library of cell lines that may reflect the genetic and maybe epigenetic diversity of a wide range of populations. Because iPSCs may develop in culture continuously, they might supply an infinite source of any required specialized cells. The ultimate objective of this technique is to employ an in vitro disease model to develop innovative medications to treat the condition (53).

Organs-on-a-chip based on iPSCs for drug screening

In vitro models, such as "organon-a-chip" (OoC) technology, have advanced as a new avenue in scientific research. OoC technology is a revolutionary approach to testing drugs for human clinical trials (54). A potential technique is iPSC-based OoC, which blends iPSC-derived 2D and 3D cell cultures with microfluidic devices. iPSC can develop as a monolayer in 2D or embedded in 3D matrices within the OoC. Major changes in proliferation, migration, differentiation, drug toxicity resistance, and gene expression were discovered when 2D and 3D cell cultures were compared (55). A microfluidic system gives mechanical and chemical physiological stimuli (e.g., compression and chemical gradients) and perfusion to the hosted cells, assuring a dynamic environment more akin to the in vivo situation. The "3Rs paradigm" (Minimize, Substitute, Refine for in vivo animal testing), for which these in vitro models have already been utilized forecast drug absorption, distribution, metabolism, and excretion and identify potential drug-induced toxicities, may be aided by the iPSCs-based OoC technology (55).

Reprogrammed iPSCs obtained from patients with diverse genetic origins can also be used to evaluate the safety and efficacy of medications in personalized medicine, where iPSCs can be cultivated on 3D matrices

within microfluidic devices utilizing techniques such as micromachining, 3D printing, and hydrogels. These circumstances reflect a novel technique that is more similar to in vivo situations (56).

An oral medication is absorbed by the gut, processed by the liver, transported to the target organs via blood flow, and eliminated by the kidneys (57). As a result, the main OoC presently being developed include the gut for absorption (58), the liver for metabolism, the kidney for elimination (59), the heart (60), the lung, the blood-brain barrier (BBB) (61), and the brain. To mimic how drugs are transported from the bloodstream to tissues and then to the target organ, the OoC must be vascularized through an endothelium. As a result, several research teams have enhanced their microfluidic apparatus for the particular purpose of drug discovery and screening by include an endothelium.

Patients' iPSC-derived intestinal organoids micro-engineered chips, which simulate inflammatory bowel illness, were utilized to examine medication absorption (62). The most prevalent reason of medication failure is hepatotoxicity caused by the medicine. Recently, drug metabolism, detoxification, and hepatotoxicity were studied on a chip using iPSC-derived hepatocytes or iPSC-derived liver organoids. Terfenadine, Tolcapone, Trovafloxacin, Troglitazone, Rosiglitazone, Pioglitazone, Lipopolysaccharide (LPS), and Caffeine, for example, were tested on a microfluidic platform equipped with a four-cell liver acinus microphysiology system comprised of PHH or iPSC-HEPs and three different human cell lines for NPCs (63).

The integration of these OoC with human iPSCs may pave the way for a new generation of OoC (dubbed patient-on-a-chip) that will permit the study of drug responses in a specific user. Fanizza et al., 2022 discussed and expanded on the role of iPSCs in drug screening for personalized medicine, particularly for neurodegenerative diseases. The translational value of OoC was investigated in order to develop more realistic disease models (64). The value of OoC has been greatly increased by the use of patient-specific iPSCs in the development of a new generation device known as "patient-on-a-chip." Furthermore, multi-OoC devices may allow crosstalk between different types of cells that replicate a genuine physiological environment extremely similar to in vivo circumstances and may be valuable in studying medication pharmacodynamics and pharmacokinetics in personalized medicine (65).

3D models of neurodegenerative diseases based on iPSCs

Brain organoids provide a new frontier in the modeling of neurodegenerative disorders. Organoids are complicated three-dimensional in vitro structures derived from pluripotent stem cells capable of self-organization and self-renewal. Organoids produced from patient-iPSCs have been utilized to examine a variety of illnesses (66). These models successfully reproduce the illnesses' main pathological markers, and several of them have been employed for drug screening studies in AD/PD (67). Shortly, the γ-secretase inhibitor DAPT, as well as heparin and heparinase, lower Aβ levels in AD-iPSC-derived cortical organoids. Compound E (Comp-E) with a BACE-1 γ-secretase inhibitor (γ-secretase inhibitor IV) decreased amyloidosis and Tau pathology in an organoid model of Alzheimer's disease (68). An organoid model of sporadic PD revealed increased caspase-3 cleavage in DA neurons in response to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahy-dropyridine (MPTP), whereas administration of the LRKK2 inhibitor GSK2,578,215A resulted in a decrease in phosphorylated-synuclein levels and improved DA neuron survival (69).

There are other non-organoid-based iPSC-based 3D static models, such as a hydrogel-based AD model that permits the investigation of early phases of Aβ oligomerization, or an AD iPSC-derived scaffold-free spheroid with a proteome profile equivalent to post-mortem AD brains (70). Rouleau et al. used an intriguing approach, developing an AD patient-derived iPSC-based 3D culture of neurons and glial cells that was stably maintained for over 2 years. Importantly, this cell culture had elevated levels of pathogenic -amyloid, Tau, and oxidative stress indicators (71). Overall, our findings show that 3D models are a powerful tool for simulating the key aspects of neurodegenerative disorders such as Alzheimer's and Parkinson's. However, 3D models have significant disadvantages, including limited repeatability and a lack of vascularization, which is required to imitate inflammation or medication distribution, both of which are important aspects in neurodegenerative diseases (72).

Conclusion and future perspectives

The current advancement in iPSC technology has created a whole new path for clinical research. However, physicians and researchers are concerned about obstacles like as irreproducibility, epigenetic changes, genetic instability, high cost, and delay. There has been considerable progress toward practical use of reprogramming methods since the first description of iPSC production. However, iPSC-based treatments are still in their infancy, with numerous obstacles to overcome before clinical applications can be realized. Individual iPSC derivation techniques have yet to be widely shown for producing cell populations for cell replacement treatment, disease modeling, and drug development, and research assessing the equivalency of different kinds of iPSCs are highly awaited. Furthermore, thorough characterization of the functioning of iPSC-derived somatic cells, as well as

their functional equivalency with in vivo counterparts, is required. The capacity to create disease-relevant somatic cells limits the application of the benefits that iPSCs provide, and considerable obstacles remain in establishing pathways that quickly lead to pure and functional populations of numerous disease-relevant cells. Given the tremendous speed of progress in the iPSC area, the capacity to take a patient's own cells, fix the disease allele, and then return those cells to the patient in a genetically and physiologically right condition is likely to be the future of customized stem cell treatment.

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Treatment of Rheumatoid Arthritis Based on Personalized Medicine: a New Approach in Rheumatology

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Abstract:
In the twenty-first century, there still needs more clarity on rheumatoid arthritis (RA). Rheumatoid arthritis is a widespread but heterogeneous illness with a broad range in its history, clinical symptoms, and response to therapy. It is now known that prevention of joint destruction, functional impairment, and a poor disease prognosis depends on early, correct diagnosis and starting therapy with disease-modifying drugs (DMARDs), among which methotrexate (MTX) remains the gold standard in the treatment of RA. Early rheumatoid arthritis diagnosis is crucial since it enables a speedier start to primary therapy. Pharmacogenetic and pharmacogenomic research, which aid in identifying a patient's genetic profile, may bring personalized treatment closer to reality. Identifying disease-specific genes while the organism's resistance to them is still intact should be made feasible by further study into RA.

INTRODUCTION

A diverse illness pattern that comprises its etiology, dynamics, course, and response to therapy will serve as the foundation for future medical practice. The guiding principle of personalized medicine is that, depending on the patient, the same illness might have a varied etiology, course, or treatment effectiveness. Consequently, it is essential to treat each patient as an individual (1).

In an era of unheard-of scientific and technical advancements, personalized medicine that emphasizes molecular diagnostics and estimating the risk of morbidity enables therapy to be tailored to each patient's requirements, increasing safety, efficacy, and cost-effectiveness. Personalized medicine is the antithesis of the conventional therapeutic method, which bases the therapy on reacting to observable symptoms of the illness. Personalized medicine is based on clinical, genetic, genomic, and environmental data unique for each patient. Personalized medicine is focused on giving the medication at the right time and in the right amount to each patient. The molecular study of not just certain illnesses but also of specific patients has made this approach practicable.

Additionally, the treatment preceded by pharmacogenetic testing is more successful since it enables the medication to be chosen based on a particular target. As a result, it is possible to predict how the patient's body will react to the medicine being administered. One benefit is increased efficacy, which is associated with a lower risk of adverse events (2). Additional advantages undoubtedly include ime saved and lower treatment costs (3).

According to the theory behind personalized medicine, diagnosing the disease at the molecular level makes it feasible to start treating patients while they are still in good condition. Genetic test results may be used to determine a person's propensity to acquire a specific illness. The relationship between genetic predisposition and increased or decreased illness risk determines how the disease will be managed. Therefore, preventive medicines should be provided to patients with a high chance of developing a disease. In order to prevent illness from developing, the patient's lifestyle should be changed (to get rid of undesirable behaviors), and regular testing should be performed (4, 5).

RA is a symptom, not a distinct illness.

RA is a long-lasting inflammatory condition affecting various joints' synovial tissue. The presence of high concentrations of acute-phase reactants, autoantibodies, and erosions on radiographs, along with the number of afflicted joints and the pattern of joint involvement, are used to make the diagnosis. Interestingly, patients with similar clinical symptoms and signs may exhibit quite different synovial leucocyte invasion and cytokine expression patterns.

There is evidence of variation in the genes linked to stromal cells, such as fibroblast-like synoviocytes, in addition to the considerable heterogeneity amongst RA patients about joint leucocyte invasion and activation of genes linked to inflammation (4, 5, 44).

Peripheral blood and synovial tissue exhibit inter-individual variation in the gene signature. For example, higher expression levels of IFN type I controlled genes have been found in the peripheral blood of nearly half of RA patients, which is compatible with the initiation of a pathogen-response program. The discrepancies between individuals with measurable anti-citrullinated peptide antibodies (ACPA) and people who are ACPA-negative substantially support the idea that RA should be seen as a syndrome comprising more than one pathogenetic entity (5, 44)

*Common final routes are impacted by efficient anti-rheumatic therapies*The fact that clinical arthritis activity is accompanied by persisting histologic evidence of synovitis after therapy with humanized anti-cluster of differentiation 52 (CD52) antibodies or chimeric anti-CD4 antibodies, despite substantial depletion of peripheral blood lymphocytes, serves as an illustration of the significance of gathering data on the synovium, the leading site of inflammation, to comprehend the effects of anti-rheumatic treatment (1, 43). Similarly, it has been demonstrated that B-lineage cells may remain in the synovium in some RA patients following rituximab treatment, even though nearly all patients significantly decrease peripheral blood B cells (2, 5, 44).

Successful use of DMARDs like gold, MTX, LEF, and CSs has consistently been linked to a reduction in the infiltration of mononuclear cells in the synovium. Like how rituximab, anakinra, and infliximab successfully treat RA patients, they also lower synovial inflammation. In one trial, patients were randomized to receive prednisolone therapy for two weeks using the COBRA regimen (Combinatietherapie Bij Reumatoide Arthritis) or a placebo. In this investigation, synovial sublining macrophages were shown to be the greatest biomarker for the clinical response to CSs. The efficacy of synovial macrophages as a possible biomarker was then evaluated across distinct therapies and kinetics (5, 44).

MEDICATIONS

Rheumatoid arthritis conventional therapy

The standard RA treatment plan comprises counseling, education, rehabilitation, and medication. Therapy aims to stop the pain, reduce or prevent inflammation, preserve normal locomotor function, delay or halt joint structural changes, and avoid organ abnormalities.

Pharmacological therapy for RA should begin as soon as feasible, ideally between 6–12 weeks after the onset of the initial symptoms. It should be successful in causing the illness to go into remission. The therapeutic window, defined as the period beginning no later than 12 weeks following the onset of the first symptoms, is the best indicator of remission (6). Patients with active RA should be checked every three months. If medication is not working, it should be changed no later than six months after starting it, according to the 2013 guidelines of the European League Against Rheumatism (EULAR).

The most crucial medications for treating RA are biological disease-modifying antirheumatic medicines (bDMARDs) and synthesized disease-modifying antirheumatic drugs (sDMARDs). Their goal is to prevent the sickness from spreading further. Nonsteroidal anti-inflammatory medicines (NSAIDs), glucocorticoids (GCs), and analgesics treat illness symptoms but do not stop the disease's progression. Figure 1 (7) illustrates the pharmacological treatment plan for rheumatoid arthritis.

Tofacitinib is a medication of the newest generation. In cases of moderate to severe symptom severity, it is used to treat active rheumatoid arthritis. This medication is marketed in 20 nations, notably Mexico,

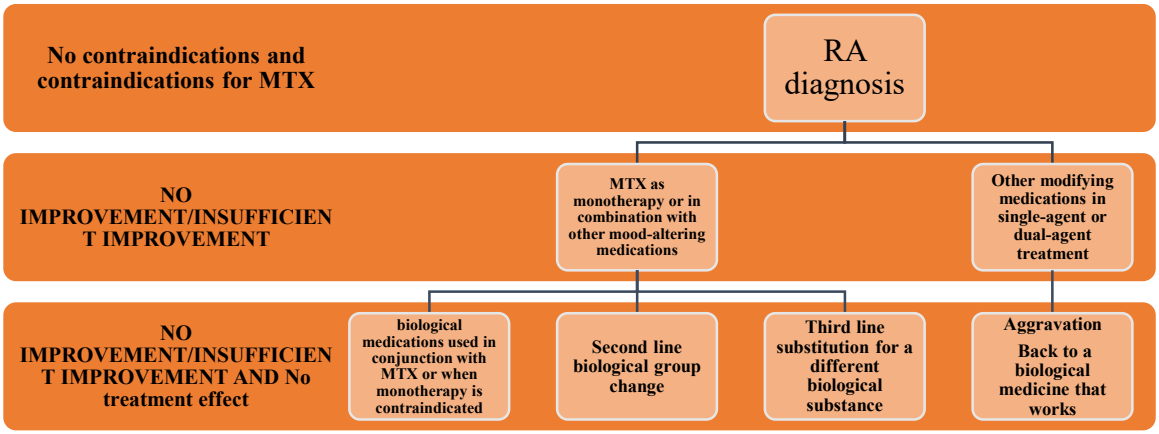

Fig 1. The regimen for treating RA with medication. Source: Original research based on EULAR suggestions (7).

Argentina, Japan, Switzerland, Russia, and Turkey. However, the European Medicines Agency has yet to approve it (EMEA). Tofacitinib must be accepted before it may be used in Poland and other EU nations.

Medications that are non-steroidal anti-inflammatory

These medications significantly shorten the length of morning stiffness and the discomfort associated with the disease's symptoms. According to European Guidelines from 2011 (8), the medication should be customized for each patient based on their potential for coronary or gastrointestinal problems.

Glucocorticoids

Glucocorticoids (GCs) and DMARDs are suggested as a first-line therapy approach in the 2010 EULAR guidelines. In the event of illness exacerbation, they are given for a brief time at greater dosages and modest doses (10 mg/day) (9). However, it should be emphasized that new research indicates that long-term usage of glucocorticoids in RA raises the chance of a heart attack by 68%; as a result, each patient's need for their administration should be carefully considered. The chance of having a heart attack after taking GCs relies on the dose, which rises by 13% with every 5 mg increase in dosage, and the period of usage, which rises by 10% annually (10). The 2013 EULAR guidelines for managing rheumatoid arthritis advise using glucocorticoids in lower dosages at the beginning of the illness and pairing them with DMARDs for no more than six months, if feasible (10).

Analgesics and pain management

The Visual Analogue Scale (VAS), the Numerical Rating Scale (NRS), and the Verbal Rating Scale should all be routinely used to measure pain in patients with arthritis, according to the recommendations of international experts in the field of rheumatology from the 3E Initiative (Evidence, Expertise, Exchange) (VRS). In the event of persistent pain, paracetamol is advised for arthritis sufferers, as is paracetamol mixed with nonsteroidal anti-inflammatory medicines. Neuromodulators that affect how pain signals are received, as well as tricyclic antidepressants, may be used to treat patients with inflammatory rheumatic illnesses. Benzodiazepines with muscle relaxants, often known as muscle relaxants, should not be used. When standard treatment fails, weak opioids may be given for a brief time. Weak opioid usage over an extended period is an option, but this treatment calls for ongoing care. Only extreme circumstances should call for potent opioids like morphine and its analogs (11).

Anti-rheumatic medications that treat disease

Synthesized anti-rheumatic medications that treat illness

Methotrexate (MTX), sulfasalazine (SSZ), leflunomide, and, in rare circumstances, azathioprine, cyclosporin A, and cyclophosphamide are examples of synthetic disease-modifying anti-rheumatic medications.

Methotrexate: Immediately upon diagnosis, methotrexate, a medication used in the initial therapy approach for RA, should be suggested. Once a week, 20–30 mg of MTX is the recommended dosage. Starting at 10-15 mg, the dosage should be raised to 20-30 mg by adding 5 mg every 2-4 weeks. Tetrahydrofolate is decreased by methotrexate via blocking dihydrofolate reductase. It prevents the metabolism of purines and pyrimidines and the creation of nitrogenous bases like thymidine. Additionally, methotrexate reduces cell growth, boosts T cell apoptosis and endogenous adenosine levels, and modifies the expression of intercellular adhesion molecules, all of which have an effect on the suppression of pro-inflammatory cytokine production and cellular response. Neutrophils, macrophages, monocytes, and dendritic cells are inhibited in their ability to cause inflammation by methotrexate.

The rise in aminotransferases activity (10-43%), gastrointestinal symptoms (20-65%), stomatitis (10-15%), anemia (10-15%), leukopenia (12%), and thrombocytopenia (12%) are among the most frequent adverse effects of MTX. Less often encountered conditions include central nervous system dysfunction (8–10%), hair loss (8%), bronchitis (2.1–8%), infectious diseases (5%), and subcutaneous lumps (2–6%) (12).

The severity of methotrexate hepatotoxicity in elderly RA patients varies with the length of treatment. Cirrhosis only affects 0.1% of individuals, but benign hepatic fibrosis affects around 7% of people. Before starting MTX treatment, the following tests should be run: liver transaminases (AST, ALT), ammonia, albumin, blood counts, and blood smears. Testing for HBsAg and HCV antibodies and a chest X-ray should be done to rule out hepatitis B and C infection. Additional options include HIV testing, glucose measurements, lipid profiles, and pregnancy tests. Control tests (AST, ALT, blood count, blood smear, and creatinine concentration) should also be performed during MTX treatment; first, every 4-6 weeks and then every 1-3 months after the target dosage has been reached. Because MTX is teratogenic, fertile women should utilize an effective means of contraception. The treatment must be stopped in women and men three months before the anticipated pregnancy. Women who are expecting or nursing cannot take methotrexate (13).

Sulfasalazine: When MTX cannot be taken or in conjunction with other medications, sulfasalazine is advised for rheumatoid arthritis treatment. Bacteria in the colon break down sulfasalazine into its two

primary metabolites, sulfapyridine, and mesalazine (5-aminosalicylic acid). SSZ's treatment of RA still needs to be fully understood. According to current research, it suppresses the production of cell adhesion proteins in leukocytes and epithelium and creates antibodies in reaction to antigen stimulation. 2-4 g/day is the therapeutic dosage for RA.

Leflunomide: Leflunomide is a prodrug whose action is dependent upon an active metabolite (A771276) produced by metabolism in the liver and intestinal walls. A771276 has a half-life of around two weeks. Interleukin 2 (IL-2) production, tumor necrosis factor (TNF) activity, antibody generation by B lymphocytes, T lymphocyte proliferation, and the migration of inflammatory cells to the synovial membrane are all inhibited by leflunomide. Leflunomide's loading dosage is one 100 mg tablet daily for the first three days of therapy. Next, a maintenance dosage of 10 to 20 mg per day is administered (depending on the severity of symptoms).

Biological disease-modifying antirheumatic drugs

Biological drugs are recommended when synthetic DMARDs (MTX in particular) have proved to be ineffective and the disease remains active. Contraindications for application or side effects of DMARDs are another reason to use biological drugs. Tumour necrosis factor α inhibitors were the first biological drugs used in treatment of rheumatoid arthritis. At present in Poland the following drugs are registered and qualified for therapeutic programmes of rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), ankylosing spondylitis (AS) and psoriatic arthritis (PsA): infliximab, etanercept, adalimumab, certolizumab pegol, golimumab.

Biologically based anti-rheumatic medications

When synthesized DMARDs (MTX in specific) have shown to be unsuccessful, and the illness is still active, biological medications are advised. Another justification for using biological medications is the presence of DMARD side effects or application contraindications.

Inhibitors of tumor necrosis factor were the initial biological medications used to treat rheumatoid arthritis. For therapy programs for rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), ankylosing spondylitis (AS), and psoriatic arthritis (PsA), the following medications are now approved in Poland: infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab.

Tocilizumab: Tocilizumab is a genetically engineered humanized monoclonal IgG1 antibody that binds exclusively to the membrane-bound and soluble interleukin-6 receptor (IL-6R) and blocks the signal transduction process by both mIL-6R and sIL-6R. 8 mg/kg of it is injected intravenously every four weeks. It is advisable to provide a dosage of less than 800 mg/infusion to individuals who weigh more than 100 kg. Both monotherapy and combination treatment with MTX is possible when using tocilizumab. The half-life of each 8 mg/kg dosage given every four weeks is between 8 and 14 days.

Abatacept: Abatacept is a soluble recombinant fusion protein made up of a human T cell-bound extracellular portion of antigen 4 (CTLA-4), linked to the immunoglobulin IgG1's modified Fc region. Abatacept blocks CD80 and CD86 molecules by attaching to CD80/86 receptors on antigen cells, which results in a reduction of the stimulating effect of CD28 protein on T cells. The recombinant DNA technique makes abatacept in Chinese hamster ovarian cells. 10 mg/kg/month is given intravenously between weeks 0 and 2, then every four weeks after that. Abatacept has a final mean half-life of about 13 days and is utilized in combination treatment with MTX or other DMARDs.

New biomarkers are required for a novel approach to managing rheumatoid arthritis

Rheumatoid arthritis still has a bad reputation in the twenty-first century. Its history, clinical symptoms, and clinical response to therapy make RA a prevalent yet varied illness. In order to avoid joint degeneration, functional impairment, and an unfavorable course of the illness, it is now understood that an early, accurate diagnosis and initiation of DMARD therapy—of which MTX represents the standard method in managing RA—are essential (14).

Patients with RA with MTX treatment failure due to toxicity or lack of effectiveness are shifted to other therapeutic choices to choose the most beneficial. Multiple ineffectual treatments come at a significant expense and may have unfavorable consequences, and it is not always feasible to have good treatment results. The right choice of a safe and effective treatment may be a useful instrument not only for treating disease symptoms (such as tiredness, joint pain, and swelling) but also for preventing joint damage, extending and improving quality of life, and even the remission of the illness. Unfortunately, even though MTX and biologic medicines typically lead to a better prognosis for RA patients, up to 40–60% of these patients fail to get a sufficient response, and 15–30% of them experience adverse medication reactions (15, 16).

Although the cause of this individual variation is unknown, studies have been able to uncover biomarkers indicative of therapy response. In the context of personalized therapy, it is inadequate to employ conventional indicators that have proved clinically helpful, including autoantibodies, acute phase reaction conditions, bone and cartilage indicators, and different cytokines (17). Therefore, according to biologists and

rheumatologists, finding new, improved biomarkers would help us understand the molecular processes involved in the pathogenesis of the illness and better choose the best medications for first-line therapy. Serum biomarkers such as proteins or microRNAs and analysis of gene profiles and gene expression patterns are likely to be more beneficial than static gene panels. We think that only by taking extensive steps to uncover biochemical, clinical, and genetic/epigenetic indicators that assist us in comprehending the many causes for focused therapy would we be able to accomplish good treatment (Fig. 2). Several variables likely influences the reaction to the effectiveness and toxicity of the treatment. However, genetic/epigenetic predisposition is one of the most significant. These biomarkers may be helpful in routine practice since they are not affected by the passage of time and may be analyzed using blood samples from patients (18). The therapeutic tactics, which are no longer a linear process, are also impacted by the understanding of the molecular basis of the illness, as is the integrated system known as the "knowledge management system," which combines clinical data from one patient with molecular and pharmacogenetic data.

Pharmacogenetics' role in genetic investigations

Genetic variations may affect illness vulnerability, disease progression, or a person's reaction to treatment, which has been shown for more than 40 years (19). Additionally, they offer many advantages over other markers, making them particularly appealing biomarkers owing to the standardized assays and linkages used to discover and confirm tests (20). In the last ten years, several studies have shown that single-nucleotide polymorphisms (SNPs) in genes that regulate the metabolism, transport, and action of pharmaceuticals may affect both the toxicity and the effectiveness of treatments (21–24).

The genes implicated in the pathophysiology of rheumatoid arthritis and the inflammatory signaling system were chosen as the candidate genes for genetic variation analysis (24). Although several SNPs are related to treatment response in RA patients, the bulk of these results still needs to be tentative and consistent. They are restricted to recognized genes implicated in the cellular pathways of DMARDs and biological drugs (21, 23). Size of samples (typically too small, n 1000), amount of SNPs in drug target genes, variations in immigrant communities, population stratified, biological background for tested populaces, variations in clinical features of patients, variations in disease stages, prior drug history, and finally variations in study design may all be contributing factors to the discrepancy (18, 23). Genome-wide association studies (GWAS) may be a more effective method for selecting potential genes for inclusion in these pharmacogenomic models since numerous genes, not just one, are implicated in the etiology of RA and medication responses (15, 22). GWAS is a practical, hypothesis-free way to find common disease-associated SNPs prevalent in the general population, scanning hundreds of thousands or millions of polymorphisms throughout the whole genome for each person (18, 25). The success of GWAS has revealed the complex genetic architecture susceptibility and opened up a sizeable new field for investigation (19). The results from GWAS and the biochemical and clinical connections between the particular loci and illnesses should be further investigated by conventional candidate gene investigations, such as allelic discriminating by TaqMan real-time PCR (18, 25). This is because GWAS do not identify the link between a gene and the disease phenotype. New methods, like next-

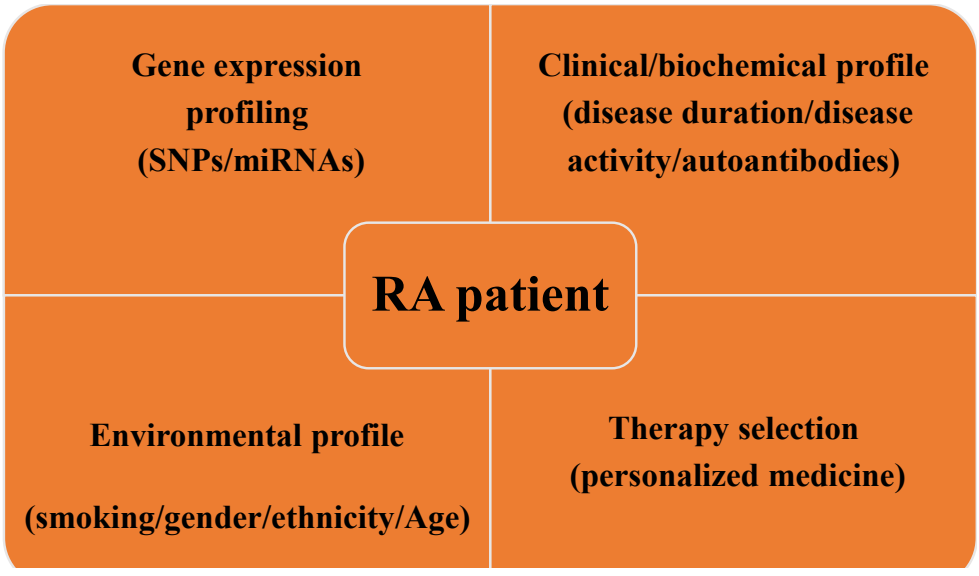

Fig2. Procedure for RA patients› treatment optimization.

generation sequencing (NGS), which are not restricted to gene chips and allow the discovery of common and uncommon variations influencing the response to medications or adverse drug responses, are being developed in addition to GWAS (18). Data produced utilizing NGS technology, which has high sensitivity and bandwidth qualities, is more persuasive than that obtained using GWAS.Furthermore, both fundamental and clinical investigations benefited greatly from the NGS analysis. Additionally, the identification of causal gene loci allows us to pinpoint elements connected to RA's pathogenesis and the effectiveness of treatment. One of the crucial stages in understanding the etiology of polygenic illnesses is gene identification. However, the research mentioned above showed that genetic variations (located in the promoter, regulatory, or coding regions of the relevant gene) contribute significantly but insufficiently to the risk of developing RA. The creation of novel intervention techniques will be made feasible by gene-expression profiles and epigenetic alterations, which are generally acknowledged to offer an extra window for understanding the potential pathways that contribute to the pathogenesis of RA.

Pharmacogenomics: the profiling of gene expression

In rheumatology, the concept of "personalized or precision" medicine, which permits the future use of genetic data for a logical choice of treatment to optimize health outcomes while reducing adverse effects, is a natural development of the knowledge we have accumulated over the years few decades. Gene-expression profiling, which examines how genes are expressed or activated, is a molecular fingerprint that has enormous promise for RA pathopathogenesis research as well as patient management and individualized therapy planning (16, 26).

Many human transcripts only express partially during a particular stage of illness, according to research on the measurement of gene expression in various tissues or situations (18). Genetic variations in regulatory elements, such as cis-expression quantitative trait loci (eQTL), which are likely active on the same chromosome, or trans-eQTL, which act on distant genes on non-contiguous chromosomes, may affect the abundance of a gene transcript (18, 27). With eQTLs, it is possible to link polymorphisms with unknown functions whose higher expressions are linked to complex traits due to pleiotropy (28, 29).

In order to comprehend the biology of RA illness and possible medication development pathways, this technique enables the discovery of correlations between genes and areas linked with the risk of RA. Genome-wide gene expression analysis using cDNA microarrays has become a powerful technique that may be used to find genes that may be biomarkers for the detection and surveillance of disease severity (30, 31). This is because genes regulate their biological functions in groups rather than alone. Because hundreds of genes were examined and expression profiles were associated with medication responses, cDNA microarrays allowed for a more thorough investigation of drug reactions. The small size of cohorts and the dynamic phenotype of RA are significant barriers to identifying reliable biomarkers. They are why prognostic and diagnostic microarrays have not been established and clinically applied, even though microarray analysis has led to identifying a gene signature that distinguishes the phase of RA and the response to therapy. NGS is currently challenging arrays as the preferred technique for genome analysis since it offers various benefits over microarrays, such as vast parallel RNA sequencing, identification of non-coding transcripts, and alternative splicing processes (16).

After all, the scientific community uses microarrays, and there are established bioinformatics processes to interpret array data. When the DNA or RNA of several specimens, such as strains isolated, has to be probed or when a low-cost "fast glance" is necessary, microarrays may be helpful as a screening tool. However, NGS might be utilized to offer thorough deep-sequence analyses of genomic DNA to detect alterations after specimens of interest are identified (32). Genome-wide methods such as NGS and microarray analysis may increase the overall survival of RA patients and increase the accuracy of diagnosis and therapy response prediction. Additionally, there are compelling arguments in favor of using a similar strategy to enhance the treatment of individuals with rheumatic disorders, given the successful use of gene-expression profile data in clinical usage in cancer.

Pharmacogenomics using microRNA profiling

The level and function of a protein may be affected by genetic polymorphisms not just in the promoter, regulatory, or coding sequence of the relevant gene, which can modify how a protein is expressed. It is commonly acknowledged that essential tasks in the control of gene expression need epigenetic processes like DNA methylation or microRNA (miRNA, miR). Additionally, recent research has shown that miRNAs are novel pharmacogenomics indicators for anti-rheumatic medications, and epigenetic aberrations are emerging as critical pathogenic aspects of rheumatoid arthritis (33). Small, noncoding RNA molecules called miRNAs tightly control biological processes by modulating protein levels at the posttranscriptional level. They comprise roughly 1-2% of the whole genome (34–36).

They serve essential immune response controllers in both healthy and diseased circumstances. In the past,

miRNAs were thought to control gene expression inside the cell. MiRNAs, however, are emerging as new candidate biomarkers for diagnosis and prognosis in a variety of diseases. These disorders, including RA, may be detected directly in organs and biological material by polymerase chain reaction (PCR) or array technology and are stably present in a cell-free state in bodily fluids like plasma or serum. In the last two years, it has been proposed that miRNAs impact the immune cell niche and regulate cellular metabolism (35). A few miRNAs were up-regulated in both plasma/serum liquids and inflamed joints; abnormal miRNA expression in individuals with rheumatic illnesses was initially described less than ten years ago (34, 35).The miRNAs are reportedly expressed even at various phases of disease development; they may also help monitor RA severity and understand its pathophysiology (33). MiR-16, -132, -146, and -155 have been revealed to have a role in regulating the growth and operations of rheumatoid-associated cells (34). MiR-146 and miR-155, which are involved in forming innate and adaptive immune cells, are crucial for maintaining immunological homeostasis and are up-regulated in inflammatory conditions. These two miRNAs in RA patients have been the subject of the most research (34, 35). Due to the negative correlation between plasma levels of the miRNAs mentioned above and disease activity measures, they serve as biomarkers for RA activity that may be helpful for therapy monitoring (DAS-28, VAS, number of tender joints (34). Although a few miRNAs contribute to various RA pathogenesis and have solid therapeutic potential, specific miRNA signatures in RA have yet to be identified. However, miRNAs can control many immunological pathways and function as messengers for cell-to-cell communication, making them perfect candidates for therapeutic development. Because of this, it is now clinically feasible to look for particular expression profiles of miRNAs in RA patients for prognostic/diagnostic reasons (34).

Therefore, therapeutically controlling miRNA levels may open up new avenues for optionally controlling the immune system and delaying or halting the course of illness. Compared to conventional pharmacological treatments, miRNA-based therapies offer a few advantages: they are a class of highly effective and selective regulators, and one miRNA may control many genes simultaneously, impacting numerous signaling pathways (35). However, a greater understanding of the roles of the previously and recently found miRNAs is required before we can envision the development of miRNA-based therapeutics for the treatment of RA.

There is a genetic test for rheumatoid arthritis.

Millions of RA sufferers take medicines every day that are useless to them. Early rheumatoid arthritis diagnosis is essential since it allows for a quicker start to the primary therapy. Pharmacogenetic and pharmacogenomic research, which helps identify a patient's unique genetic profile, may advance personalized medicine. Rheumatologists still hope to find precise biomarkers for diagnosing and treating RA. Unfortunately, pharmacogenetic/pharmacogenomic testing is not widely used nowadays. This may be the result of several factors, including the heterogeneity of RA, incomplete knowledge of the disease's pathogenesis, a small sample size, and other non-genetic factors (demographic, environmental, and clinical or serological markers) that can affect or predict a drug's efficacy or toxicity in RA patients.

Furthermore, there are several reasons why it is crucial to pinpoint the specific genes and epigenetic modifications responsible for the onset, progression, and therapeutic response to rheumatoid arthritis. These include forecasting who will acquire RA, estimating the severity of the condition, predicting how an individual with RA will react to therapy, and discovering novel therapeutic targets. Identifying how genetic/epigenetic changes impact the biochemical function in specific cell subtypes and are related to RA susceptibility and severity will be one of the biggest problems for researchers in the following years. Integrating the most recent findings from genetic testing into clinical practice by creating assessment tools that benefit from personal genomic/epigenetic information (18). Recent developments in cutting-edge technology, like NGS, could provide a more individualized approach to patient practice, with improved risk classification and treatment options based on data from a unique genetic/epigenetic background (18). Though companion diagnostics may not be cost-effective, pharmacogenetic/pharmacogenomic approaches may be costly due to the high cost of biologic therapy for RA patients. Additionally, identifying the genetic/epigenetic elements behind the variation in medication treatment responses may help identify responder and non-responder individuals early, encouraging the development of better and more efficient therapeutic approaches for rheumatoid arthritis patients.

Therapy selection

Treatment can be tailored based on variations in treatment response and clinical phenotype-based personalization. The best impact lasts at least 6 to 12 weeks, so selecting the proper DMARD medication is crucial to achieving the suggested therapeutic objectives. As a result, it is best to forecast the outcome of the therapy before it begins. Future rheumatologists will likely use biomarkers to guide their treatment decisions, particularly when deciding between bDMARDs and tsDMARDs. For instance, Tao et colleagues utilized machine learning to

create models based on gene production and DNA methylation information to forecast how RA patients will respond to adalimumab and etanercept. When utilizing gene expression, they obtained a response prediction accuracy of 84.7% (adalimumab) and 88% (etanercept), and when using DNA methylation, they reported reliability of 85.9% (adalimumab) and 79% (etanercept) (45).

Future of personalized medicine

The likelihood that rheumatoid arthritis may proceed to pathology decreases the sooner the correct diagnosis is obtained. Further research into RA should make it possible to identify disease-specific genes while the organism's resistance to them is still intact (before auto-aggression develops). Future research should focus on understanding how tolerance is broken in spatiotemporal in vivo networks with the benefit of rationalizing current treatments or giving the correct patient the proper medication at the right time and place (37).

Because around one-third of RA patients do not react to a particular biological treatment, tailored medicine is crucial for RA patients. The progression of RA is quite complex. This likely explains why every patient responds to therapy differently (38). Studies conducted on RA patients do not account for distinct pathotypes responsible for therapy response. According to British research, a poor response to TNF inhibitors and a high degree of impairment in the RA patient population are strongly correlated (39).

Lack of concurrent treatment with MTX or non-steroid anti-inflammatory medications reduces the likelihood of response, particularly to etanercept. Also seen in the group of women is a lower likelihood of remission. Swedish trials' findings show that individuals treated with DMARDs and with a lower degree of impairment have better success in TNF inhibition. The results of the Danish investigations, however, indicate that older patients and those who had prednisolone had a lower response to the initial anti-TNF medication (39).

Future customized therapy for rheumatoid arthritis should be based on a "composite grading system" based on several biomarkers and demographic data. Consequently, the symptoms might adapt to each patient's therapy individually. This will lessen adverse effects, enhance results, and save expenditures. Studies comparing various biological treatments "head-to-head" that can suggest the best therapeutic choice is progressively taken into account. For instance, some study findings suggest that tocilizumab may be a better initial option for individuals who cannot tolerate MTX while receiving biological treatment. These data must, however, be evaluated in light of the carefully chosen clinical trial group (38).

Anti-citrullinated protein antibodies are used in new diagnostic criteria for rheumatoid arthritis that have been developed (ACPA). The next stage is identifying the indicators that may be utilized to discriminate between RA and undifferentiated arthritis. The discovery of biomarkers at this time and the creation of tools that include biomarkers and stage-related clinical features will influence the beginning, choice, and length of therapy (40). Early diagnosis and prompt treatment are crucial for inducing remission and preventing irreparable damage to the joints in rheumatoid arthritis. Preferably, an initial detection should be made during the asymptomatic or pre-clinical stage. According to several studies, the rheumatoid factor (RF) and ACPA were both present before the onset of RA (41).

In clinical practice, tests that measure well-known diagnostic biomarkers are often utilized. According to estimates, the outcomes of these tests form the basis of 70% of the treatment choices made by doctors. However, integrating new biomarkers into medical care has proven to be a protracted and challenging process involving persuading doctors. A crucial stage in ensuring the biomarker's adoption in medical care and further improvement of its usage is evaluating its effect on general health. As biomarkers are used more often in clinical practice, this study topic is becoming more and more significant. The complexity and heterogeneity of rheumatoid arthritis make it unlikely that a particular cytokine could distinguish between different types of the disease well enough. Several trustworthy multiplex cytokine tests are now leading in this field (although, in the case of RA, it may not be an appropriate solution due to RF interferences). Since tests are helpful in the treatment of other illnesses, implementing them in rheumatology should be simple (technically). However, it is essential to define the precise performance definition and quality control for the relevant cytokines in RA. The disease's intricacy now constrains RA as it relates to cytokine networks. Future customized treatment for RA may take a more practical approach if it uses various biomarker profiles based on genetic and proteomic markers. A multivariate study like this one could show trends rather than specific biomarkers. A single IL-7 may predict diagnosis in the early stages of the illness. However, a more complicated mix of markers may be required to predict the response to treatment and identify subgroups of individuals with more progressive disease (42).

CONCLUSION

In order to increase responsiveness, maintain the structural and functional characteristics of the joints, and lower treatment costs, there is a critical need for reliable biomarkers that relate to the reaction to

biological therapy. According to certain studies' findings, it is now possible to forecast how effectively rituximab will work as a therapy because of various clinical traits related to how the body will react to TNF suppression and the existence of antibodies in the blood. A lack of reaction cannot be foreseen, but current response markers may forecast the likelihood of response to a medicine or the quality of response. Antibodies play a significant role in the new ACR/EULAR clinical definition for rheumatoid arthritis. Including seropositive individuals in RA cohorts in the future cannot be ruled out. So far, treating patients with seronegative RA with another drug makes sense before starting them on rituximab (39, 43).

Before molecular illness diagnosis—the cornerstone of the tailored approach—becomes the norm, personalized medicine must address three fundamental problems. The first and most crucial problem is the need to test one million single nucleotide polymorphisms (SNP) that are present in the genome to determine genetic diversity is the first and most crucial problem. The SNPs that cause the illness and may serve as therapeutically useful indicators should next be identified. Another concern would be money-related issues. Only costly genotyping techniques and a thorough grasp of biological defense processes make it feasible to search for disease indicators. Another problem is that protein markers are challenging to type since there is restricted access to the proper tissues in the case of numerous disorders. For this sort of data analysis to be successful, both proteomic and computational technologies need additional development.

Finding reliable biomarkers, such as genetic markers, is necessary to forecast uncommon adverse occurrences. These markers ought to be capable of being detected in small samples. However, there is precedence for this. For instance, thiopurine S-methyltransferase genetic polymorphism and azathioprine-induced bone marrow reduction (40) or liver toxicity from flucloxacillin and HLA-DRB*5701 (OR > 80) are examples.

Biomarkers must be found and verified to apply for tailored medicines as effectively as feasible. Therefore, it is necessary to create new rules that outline how industry and academics interact regarding regulatory oversight.

New norms for stakeholders participating in all phases of the implementation of personalized medicine must also be developed, from the validation of biomarkers to the patient's informed consent. Various barriers are preventing customized medicine from being widely adopted. The actual example is the need for specified norms of behavior and stakeholder participation and the inappropriateness of European financing policies and data availability. Another obstacle to the adoption of customized medicine is the healthcare system. These issues can and ought to be successfully resolved. Systematic steps must be promptly done to overcome obstacles to customized medicine's implementation in order for it to be used successfully (44).

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Feature	Description
Excitation Source	8 dye specific LEDs per optical module
Detection Sources	8 photodiodes
Optical Cartridges	SYBR/FAM HEX ROX CY3 CY5 ATTO425 6 slots, swappable optical modules
Dye Selection	Excitation and Emission
Reaction Volume	10 µL to 30 µL
Chemistries Supported	SYBR, Probe, HRM
Thermal System	Six Peltiers made from two ceramic plates with semi-conductor elements, 96-well
Thermal System Temperature Range	25.0 – 99.9°C Heating: 6.0°C/sec Cooling: 3.0°C/sec (Median), 2.5°C/sec (Average) Accuracy: ± 0.2°C or better at typical annealing, amplification, and denaturation temperatures
Dynamic Range	9
Experiment Types	Quantitative PCR with dye, Quantitative PCR with probe, Allele Discrimination with HRM, Allele Discrimination with probe, Comparative Quantitation, User Defined
Uniformity	± 0.4°C
Data Acquisition Time	<3 seconds for all
Cq Uniformity	Cq St Dev <0.20 at fast cycling (5s 95°C/10s 60°C)
Electrical Power (input)	100 – 240VAC, 50/60Hz, 1100VA
Operating Environment	20 – 30°C, 20 – 80% non-condensing humidity, 7500 feet, max altitude
Weight	50 lbs. (23 kg)
Dimensions	19.7" W x 18.1" D x 16.5" H (50cm x 46cm x 42cm)

Feature	Description
Sample Containers	96-well plates, strip tubes; 0.2 mL tubes
Warranty	• 1-year warranty is standard with the instrument • 5-year warranty and service packages available
Onboard Analytics	• Thermal, physical, interactive (sensors) tests • Extended: 125 performance points tested in 30 minutes • Start-up: 59 performance points tested in ~ 1 minute • Optional bypass of both features
Services (upon request)	• Installation and familiarization • Standard and Enhanced Preventative Maintenance • Additional year warranty (+1 increments, up to 5 years coverage) • Return-to-Agilent Instrument Exchange Program • Thermal block verification
Operating System	• Windows 7 and 10
MS Office Compatibility	• Microsoft 2010 and 2013 compatible
Run Modes	• Stand alone • PC connected • LAN connected to PC (more than 20 instruments can be connected and monitored remotely) • USB connected, external devices
Software	Free software including LIMS connectivity
Optical Module Calibration and Cleaning	• All channels can be tested and calibrated • All attributes of optical channels are calibrated at the factory – LED light output, light path, mirror, and photodiode • Optical modules can be cleaned in lab without Agilent technician or sending back to factory
Selected Applications	• Quantitative and qualitative gene expression analysis • miRNA analysis • Genetic mapping • Genetic fingerprinting • NGS library quantification • 2-6 channel multiplex ability • HRM analysis (including genotyping, mutational analysis, and class IV SNP detection) • Pathogen quantification

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