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SCHOOL OF MEDICINE

Department of Health Sciences

Master degree in Medical Biotechnologies

Master thesis

Association Between a MerTK Gene Polymorphism (rs4374383) and Major Autoimmune Diseases in a Clinical Cohort: Focus on Rheumatoid Arthritis

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1. Abstract

Background:

Autoimmune diseases are chronic immune-mediated conditions characterized by a loss of self-tolerance and ongoing inflammation. Genetic variation in immune-regulatory genes likely contributes to differences in disease distribution and overlap among patients. MerTK, a tyrosine kinase receptor involved in immune homeostasis and inflammation resolution, has been linked to autoimmune regulation.

Objective:

The aim of this study was to assess the association between a MerTK gene polymorphism and the occurrence of major autoimmune diseases within an autoimmune patient group, particularly focusing on rheumatoid arthritis (RA).

Methods:

This observational cohort study enrolled adult patients with autoimmune diseases. Peripheral blood samples were analyzed to extract genomic DNA and determine the MerTK polymorphism using RFLP-PCR. Genotype frequencies were calculated and tested for Hardy–Weinberg equilibrium. Pearson's chi-square test assessed differences in genotype distribution. Univariate logistic regression further explored associations between the polymorphism and major autoimmune conditions. A two-sided p-value < 0.05 was considered statistically significant.

Results:

Seventy-one patients were included (73.2% female; median age 57 [49–68] years). Rheumatoid arthritis was present in 50.7% of patients, autoimmune hepatitis in 38.0%, interstitial pneumonia in 31.0%, thyroiditis in 14.1%, and type 2 diabetes mellitus in 14.1%. Genotype distribution was G/G 33.8%, G/A 49.3%, and A/A 16.9%, and was consistent with Hardy–Weinberg equilibrium ($p = 0.437$). No statistically significant difference in genotype distribution was observed between patients with RA and those without ($\chi^2 = 2.03$, $p = 0.362$). Logistic regression analysis did not demonstrate significant associations between the MerTK polymorphism and RA or other evaluated autoimmune conditions (all $p > 0.05$).

Conclusion:

In this cohort of patients with autoimmune diseases, the investigated MerTK polymorphism was not significantly linked to major autoimmune conditions. This suggests the genetic variant does not seem to significantly influence the distribution of autoimmune disease within the studied population.

2. Introduction

2.1 Autoimmune Diseases and Rheumatoid Arthritis (RA)

Autoimmune diseases are a heterogeneous group of chronic disorders characterized by the loss of immune tolerance and inappropriate immune responses to self-antigens. This results in persistent inflammation and progressive tissue damage. They affect around 7–10% of the global population and encompass both organ-specific and systemic conditions like rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes, and multiple sclerosis (1). These clinically diverse conditions share a common pathogenic framework, involving the convergence of genetic susceptibility, environmental triggers and immune dysregulation. Genome-wide association studies have identified hundreds of shared risk loci, particularly in the HLA region. This suggests a polygenic architecture and significant overlap in genetic determinants across different autoimmune phenotypes (2). Notably, the majority of susceptibility variants are located in non-coding regulatory regions, underscoring the crucial role of transcriptional and epigenetic regulation in disease development. Beyond genetic predisposition, epigenetic mechanisms such as DNA methylation, histone modification and chromatin remodelling significantly influence immune cell function, contributing to disease heterogeneity and progression (3).

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that causes persistent inflammation of the synovial tissue and gradually damages the joints over time. In most cases, the inflammation occurs bilaterally and can progressively spread to multiple joints, leading to stiffness, swelling, reduced range of motion, and long-term disability if left untreated. Moreover, synovial inflammation is associated with the formation of the pannus, a hypertrophic synovial tissue contributing to cartilage destruction, bone erosion, and joint dysfunction. Additionally, it may present with extra-articular manifestations (EAMs) affecting various organs and tissues, including the heart, kidneys, lungs, gastrointestinal tract, eyes, skin, and nervous system (4–7). Patients' quality of life can be significantly affected by these systemic features, and, in some cases, may even precede the onset of articular symptoms.

The global prevalence of RA ranges between 0.5% and 1%, with considerable variation based on geographic, ethnic, and socio-demographic factors (4–6,8,9). According to epidemiological studies, RA is more prevalent in women, who are affected approximately two to three times more often than men, possibly due to hormonal and immunological differences. Individuals with a

family history of autoimmune diseases and those who smoke are also at increased risk of developing RA. The disease commonly begins between the ages of 40 and 60 and follows a symmetrical polyarticular pattern, often evolving from vague constitutional symptoms such as fatigue or malaise to more specific joint-related complaints as the disease progresses (9,10) . **Figure 1** shows the systemic complications of rheumatoid arthritis.

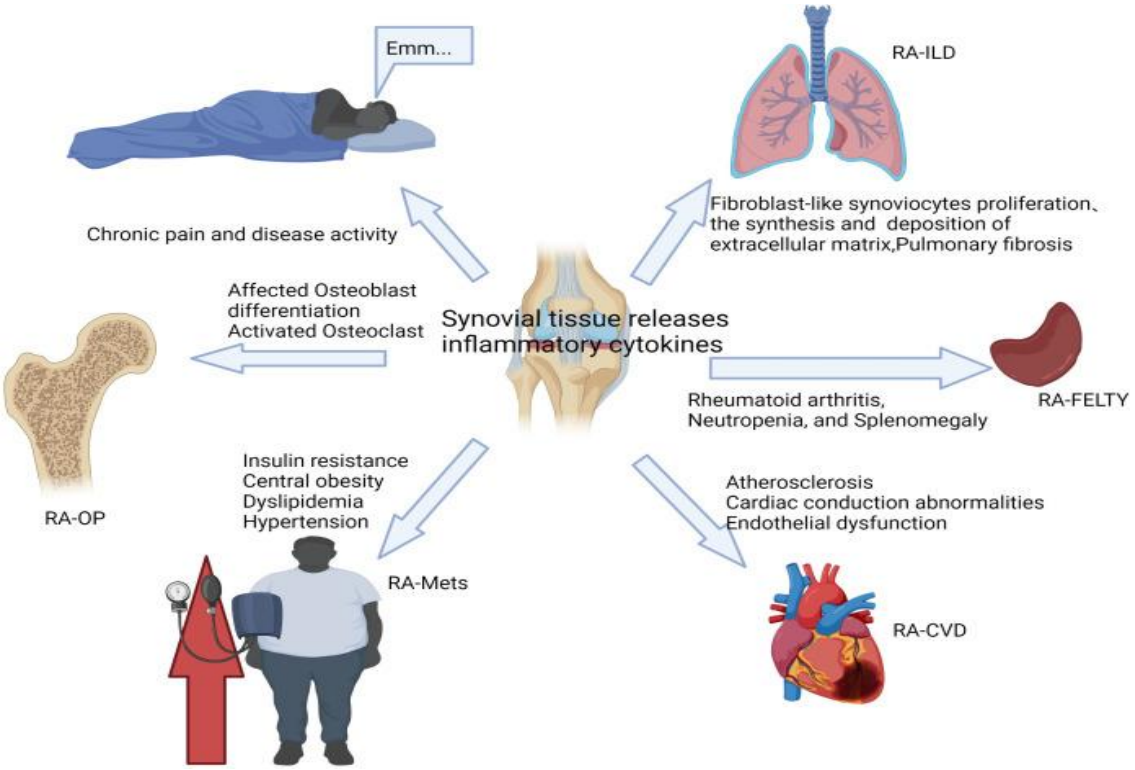

Figure 1: Systemic complications of rheumatoid arthritis (RA). Chronic synovial inflammation drives cytokine release, which contributes to multi-organ involvement including interstitial lung disease (RA-ILD), Felty's syndrome, cardiovascular disease (RA-CVD), metabolic alterations (RA-Mets), osteoporosis (RA-OP), and chronic pain (6).

The pathogenesis of RA involves a complex of interactions between HLA and non-HLA genes, as well as immunological and environmental factors. HLA-DRB1 has the strongest effect on RA progression, especially within HLA class II. There are some risk alleles that have been linked with this gene which encode a specific amino acid sequence called the shared epitope (SE), located at

positions 70–74 of the HLA-DRβ chain. The presence of the shared epitope within the HLA-DRB1 gene disrupts normal antigen presentation and promotes autoimmunity by activating self-reactive T cells. This mechanism is particularly relevant in seropositive RA and correlates with a gene-dose dependent increase in disease risk. The presence of a shared epitope in the HLA-DRB1 gene can disrupt the normal process of antigen presentation to immune cells. Instead of presenting only true viral and bacterial antigens, this disruption causes self-antigens to be mistakenly presented as foreign antigen, autoreactive T cells become activated and attack the body's own tissues (5,11,12). This phenomenon is especially common in individuals who are Anti-citrullinated peptide antibodies (ACPA)-positive (seropositive RA). Also, the greater the number of SE alleles (i.e., one or two copies), the higher the risk of developing RA (9). Apart from HLA genes, several non-HLA genes have been associated with an increased risk in RA susceptibility. Notably, PTPN22 gene encodes an enzyme that regulates T-cell receptor signaling, a missense variant (R620W) has been associated with both reduced immune tolerance and enhanced autoreactivity. Additionally, gene mutations in CTLA4 and PADI4 are related to RA, affecting T-cell co-stimulation and post-translational citrullination of proteins, respectively. Although the effect size of these non-HLA genes is modest compared to HLA-DSB1, their cumulative contribution, especially when combined with environmental risk factors, significantly increases RA risk (11,12).

Environmental factors play a critical role in the initiation of rheumatoid arthritis (RA) in individuals who are genetically predisposed to the disease. **Figure 2** shows the factors contributing to the development of rheumatoid arthritis. Smoking, air pollution, gum disease, and microbial imbalances in the gut and oral cavity are among the best-known environmental triggers that, over time, can disrupt immune system balance and push the body into a chronic inflammatory state (11–15). These factors, in genetically at-risk individuals, may cause alterations in how proteins are processed, particularly through post-translational modifications such as citrullination and carbamylation. These modifications subtly but significantly change the native structure of proteins. As a result, the immune system may mistakenly recognize these altered self-proteins as foreign agents. This misidentification leads to a loss of immune tolerance, where the body can no longer distinguish between self and non-self, resulting in the activation of autoreactive B and T cells. This misdirected immune response contributes to the production of autoantibodies such as ACPA (anti-citrullinated protein antibodies), which are particularly prevalent in seropositive RA patients. Over time, these autoimmune attacks cause chronic inflammation in synovial tissue and progressive

joint destruction. Furthermore, this cascade is often exacerbated when genetic predispositions (such as shared epitope alleles of the HLA-DRB1 gene) and environmental triggers interact simultaneously, creating the so-called perfect storm for RA development (5,10,16).

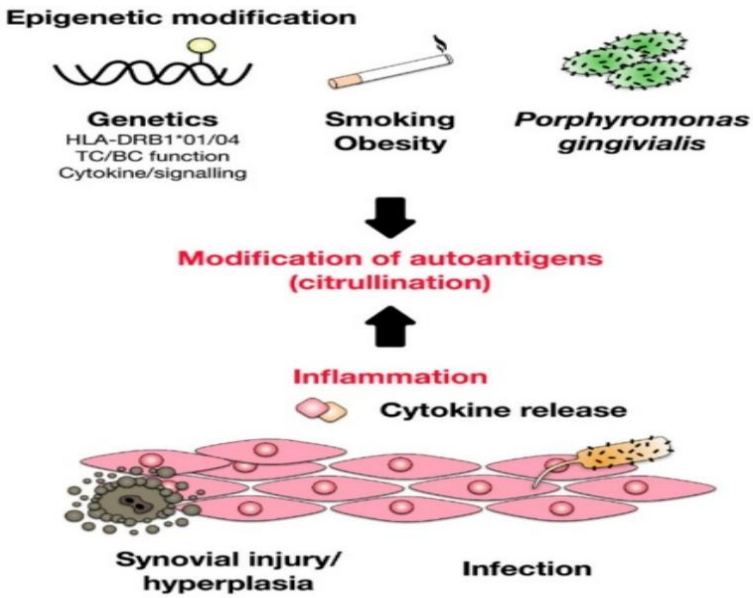

Figure 2: Factors contributing to the development of rheumatoid arthritis (RA). Both genetic predisposition (e.g., HLA-DRB1) and environmental triggers such as smoking, obesity, or infection with Porphyromonas gingivalis can promote synovial inflammation, leading to citrullination of autoantigens and subsequent joint damage (8).

2.2 Autoantibodies in RA

As stated previously, RA is an autoimmune disease characterized by an aberrant immune response targeting the synovium, resulting in chronic inflammation and joint destruction. The autoimmune nature of RA is driven by the presence of autoantibodies and an aberrant immune response that targets the body's own tissues. The identification of autoantibodies, such as rheumatoid factor (RF) and ACPA, is routinely performed in patients with symptoms of inflammatory arthritis (6,9,12). RA patients having these autoantibodies are defined “seropositive”, while patients who have clinical symptoms of RA but lack these antibodies are known as “seronegative”. Seronegative RA patients are thought to be less prone to disease progression and are generally easier to treat (10). RF was the first autoantibody identified in RA, produced by B cells that accumulate in lymphoid follicles and germinal center-like structures within the inflamed synovial membrane. This antibody

binds to the Fc region of IgG immunoglobulins and may be represented by an IgA, IgM, and IgG isotypes. RF sensitivity has been reported to be between 60-70%, and specificity between 50-90%. RF can also be detected in many infectious and autoimmune diseases other than RA, and even in healthy individuals (9,12). In healthy individuals, RF is likely involved in the clearance of immune complexes by binding to antibodies that have previously bound to invading pathogens; conversely, in RA patients, the presence of RF can cause immune complex formation, activation of the complement system and macrophages, and recruitment of leukocytes to inflamed joints. Although RF has limited diagnostic specificity, it can be useful in combination with other markers in the early identification of patients and determining the need for targeted therapies (9). Another well-known type of autoantibody in RA is represented by ACPA, which is produced against proteins that have undergone the citrullination process. In the citrullination process, in inflammatory conditions, the amino acid arginine is converted to citrulline by the PAD enzyme, especially PAD2 and PAD4, which are overexpressed in immune cells. The test for ACPA was first developed in the 1990s and the specificity of this test increased to 99% (8,9,12). The presence of ACPA is associated to a more severe phenotype of RA, characterized by a higher burden of bone destruction and chronic joint inflammation. Another biomarker being investigated to better identify seronegative RA patients is the anti-carbamylated protein (anti-CarP) antibody, which targets proteins that have undergone carbamylation. In this process, the amino acid lysine is converted to homocitrulline, a molecule structurally similar to citrulline, by reaction with cyanate. The severity of this process is directly related to conditions such as inflammation, smoking, or renal failure that have higher urea levels (9). The effect of this antibody is associated with progressive joint destruction, especially in ACPA-negative patients. With a diagnostic specificity of about 45% sensitivity, and about 90% specificity, it becomes up to 98% specificity when combined with ACPA and RF (9,12). On the other hand, although they have functional similarities with ACPA, they follow an independent immunological pathway and can be useful as complementary markers for early diagnosis and assessment of RA disease severity, especially in seronegative cases (9)(12).

2.3 Chronic inflammation in RA

The chronic inflammation of the synovial membrane (synovitis) in RA is driven by the infiltration of immune cells, involving both innate and adaptive immune responses. The persistent inflammation causes the proliferation of synovial resident stromal cells (synoviocytes), leading to thickening of the intimal layer. Additionally, the formation of new blood vessels supports the continuation of the inflammatory process by allowing more inflammatory cells to enter from the systemic circulation. More specifically, the key players in this process are T cells, B cells, macrophages, and fibroblast-like synoviocytes (FLS) (5,6,8,17). FLS, which normally contribute to the structural integrity of synovium, become activated in RA and take on a pro-inflammatory role and promote inflammation and joint degradation. These cells not only produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, but also participate in the destruction of cartilage and bone by releasing matrix metalloproteinases (MMPs), including MMP-1, MMP-3, and MMP-13, which degrade extracellular matrix components and facilitate the expansion of the pannus (18). In addition to FLS, macrophages play a pivotal role in driving inflammation by secreting cytokines that amplify the immune response and recruit more immune cells to the affected joints (5,6,17). These macrophages, upon stimulation by Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) or toll-like receptor ligands, also express high levels of IL-1β, IL-12, IL-23, and TNF-α, creating a proinflammatory milieu that contributes to chronicity of the disease. The process begins with the activation of antigen-presenting cells, such as dendritic cells, which interact with T-cells to initiate an adaptive immune response. This leads to further recruitment of inflammatory cells from the systemic circulation, including T-helper cells and B-cells (19). These lymphocytes are crucial not only for cytokine production but also for promoting the production of autoantibodies, including the RF and ACPA, which contribute to immune complex formation and tissue damage. The ongoing inflammatory cascade induces the proliferation of synovial resident stromal cells, causing thickening of the intimal layer of the synovium. Concurrently, the formation of new blood vessels facilitates the continuous infiltration of immune cells, perpetuating the cycle of inflammation. This vascular expansion is largely driven by pro-angiogenic factors such as vascular endothelial growth factor (VEGF), which is upregulated in response to the inflammatory milieu. Hypoxia, common in inflamed joints, further enhances VEGF expression, contributing to neovascularization and sustained cellular influx. The resulting hyperplasia of the synovial lining leads to the formation of pannus, a thickened, inflamed tissue

that invades and damages surrounding cartilage and bone (20). A critical downstream consequence of this pro-inflammatory environment is the promotion of osteoclastogenesis, which is central to bone erosion in RA. The differentiation and activation of osteoclasts the primary bone-resorbing cells are tightly regulated by the receptor activator of nuclear factor-kappa B ligand (RANKL), a member of the TNF superfamily (5). RANKL is produced by activated T cells, B cells, and notably, by FLS in RA joints. Upon binding to its receptor RANK on the surface of osteoclast precursors, RANKL initiates signaling cascades that lead to osteoclast maturation, survival, and activation. The critical balance between RANKL and its decoy receptor osteoprotegerin (OPG) determines the extent of bone resorption. In RA patients, the RANKL/OPG ratio is significantly tilted in favor of RANKL, tipping the balance toward pathological bone erosion. This RANK/RANKL interaction is further amplified by inflammatory cytokines such as IL-1β and TNF-α, which upregulate RANKL expression and sensitize precursors to osteoclastogenic signals. Mature osteoclasts secrete proteolytic enzymes like cathepsin K and MMP-9, which degrade both the organic and inorganic components of bone matrix, leading to subchondral bone erosion. These osteoclasts are often located at the interface of the pannus and bone, emphasizing their central role in joint destruction. Moreover, activated FLS contribute to osteoclastogenesis not only via RANKL secretion but also by direct interaction with osteoclast precursors through cell-cell contact and through secretion of IL-6, IL-8, and GM-CSF, reinforcing their role in bone destruction (17). Furthermore, Th17 cells a highly pathogenic T-cell subset enriched in RA synovium produce IL-17, a cytokine known to stimulate FLS to express more RANKL and inflammatory mediators. IL-17 also synergizes with TNF-α and IL-1β to enhance osteoclastogenic pathways and suppress osteoblast-mediated bone formation, thereby creating a destructive imbalance. The pathogenic triad of Th17 cells, FLS, and osteoclasts forms a vicious cycle of inflammation and bone degradation that underpins the erosive nature of RA (5,19). In addition to enhancing bone resorption, chronic inflammation suppresses new bone formation. Pro-inflammatory cytokines inhibit osteoblast differentiation by upregulating Wnt pathway antagonists such as Dkk-1 and sclerostin, which block osteoblastogenesis and bone repair. This imbalance between resorption and repair contributes to irreversible joint damage in patients with longstanding RA (5,8). In conclusion, the chronic inflammation in RA is far more than just an immune response; it is a coordinated, self-amplifying process involving immune cells, structural cells, and cytokine networks that converge on the RANK/RANKL axis to drive bone erosion. This dynamic interplay

not only explains the characteristic joint destruction in RA but also offers therapeutic opportunities. Targeting the RANKL pathway (e.g., with denosumab), modulating FLS activation, blocking IL-17 signaling, and restoring the balance between osteoclasts and osteoblasts are emerging strategies that may provide both symptom relief and structural preservation in patients with RA.

2.4 Clinical manifestations and therapeutic approach

Clinically, RA is diagnosed based on a combination of symptoms, serological tests (RF, ACPA), and imaging findings such as musculoskeletal ultrasound and magnetic resonance imaging (MRI). To standardize the diagnosis, the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria are widely used. These criteria assign weighted scores based on the number and size of joint involvement, serological findings (RF and ACPA), acute-phase reactants (C-reactive protein and erythrocyte sedimentation rate), and symptom duration. A total score of six or more out of ten confirms classification as RA (10,21). The treatment strategy for RA is now guided by a treat-to-target (T2T) approach, which emphasizes early and aggressive intervention to achieve remission or low disease activity. The cornerstone of pharmacological therapy begins with conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), with methotrexate being the first-line agent due to its efficacy, long-term safety, and affordability (5,22). In patients with insufficient response to methotrexate, the addition of biologic disease-modifying antirheumatic drugs (bDMARDs) is recommended. These include tumor necrosis factor (TNF) inhibitors such as adalimumab, infliximab, and etanercept; interleukin-6 (IL-6) receptor antagonists such as tocilizumab and sarilumab; the anti-CD20 monoclonal antibody rituximab; and T-cell costimulation blockers like abatacept. Each targets specific immunological pathways implicated in RA pathogenesis (5,22–24). In recent years, targeted synthetic DMARDs (tsDMARDs), particularly Janus kinase (JAK) inhibitors such as tofacitinib, baricitinib, and upadacitinib, have emerged as potent oral alternatives. These agents disrupt intracellular signaling pathways critical for cytokine activity and are especially useful in patients unresponsive to bDMARDs or those with contraindications (4,22). Despite therapeutic advancements, a significant proportion, about 20–40%, of patients fail to achieve optimal disease control and are classified as having difficult-to-treat (D2T) RA. This clinical challenge underscores the need for individualized treatment algorithms, regular monitoring, and a multidisciplinary

approach to care (5,22,24). Ongoing research into disease mechanisms and treatment response has led to a growing emphasis on precision medicine in RA. Multi-omics technologies, including transcriptomics and single-cell analyses, are providing valuable insights into disease heterogeneity and guiding patient stratification for personalized interventions (5,24,25). Novel biomarkers, particularly non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), are being evaluated for their potential roles in early diagnosis, prognosis, and predicting therapeutic response (5,15,16). Understanding the immunological evolution of RA has also revealed a preclinical phase marked by systemic autoimmunity that may begin years before clinical symptoms emerge. Identifying at-risk individuals during this phase opens new avenues for preventive interventions and early treatment strategies (8,13,26).

2.5 TAM Receptors: Structure and Immune Functions

The TAM tyrosine kinases (Tyro3, Axl, and MerTK) receptors comprise a unique Receptor Tyrosine Kinases (RTKs) subfamily, discovered in the early 1990s, distinguished by an extracellular domain of two immunoglobulin-like and two fibronectin type III repeats and activated by the vitamin K-dependent ligands growth arrest-specific protein 6 (Gas6) and Protein S (27). TAM signaling has pleiotropic roles in normal physiology, governing cell survival, proliferation, differentiation, migration, tissue and immune homeostasis, and apoptotic cell clearance, while loss of TAM activity results in aberrant inflammation, autoimmunity, and tumorigenesis (28). TAM receptors are indispensable for proper resolution of inflammation, as they mediate efferocytosis (apoptotic cell clearance), suppress excessive innate immune responses, and promote restoration of vascular integrity; correspondingly, TAM deficiency leads to uncontrolled inflammation and systemic autoimmunity (29). MerTK activation in macrophages also stimulates the production of specialized inflammation-resolving mediators via suppression of Calcium/Calmodulin-dependent protein kinase II (CaMKII) signaling, illustrating how TAM signaling actively drives the return to homeostasis after immune challenge (30). Moreover, TAM engagement in macrophages dampens proinflammatory signaling (through Toll-like and cytokine receptors), induces autophagy to limit NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation, and skews macrophage polarization toward a reparative M2-like state, thereby broadly curbing inflammatory responses (31). The Gas6/TAM axis also influences chronic tissue remodeling, linking unresolved inflammation to fibrogenesis and tissue scarring in multiple organs such as the liver, lung, and kidney during persistent injury (32). In the

nervous system, TAM receptors regulate neurodevelopment and repair processes (e.g., neurogenesis, synaptic plasticity, microglial phagocytosis, and myelination), and TAM dysregulation has been implicated in neurodegenerative diseases such as Alzheimer's and Parkinson's, as well as in demyelinating conditions like multiple sclerosis (33). TAM are frequently upregulated in cancers, where they drive oncogenic signaling that promotes tumor cell survival, invasion, metastasis, angiogenesis, and therapy resistance, and their high expression often correlates with poor patient prognosis (34). In cancer cells, TAM receptors can also signal in a ligand-independent manner by acting as co-receptors for other oncogenic RTKs (e.g., mutant Fms-like tyrosine kinase 3 (FLT3) in leukemias), and TAM activity has been linked to immune checkpoint pathways, underscoring a pivotal role in tumor immune evasion (35). Within the tumor microenvironment, TAM signaling in macrophages and other myeloid cells fosters an immunosuppressive milieu that enables tumor growth and resistance to therapies, emphasizing the interplay between TAM-mediated immune regulation and cancer progression (36). Consequently, TAM kinases (notably AXL and MerTK) have emerged as viable therapeutic targets, and multiple TAM-directed agents (small-molecule inhibitors and monoclonal antibodies) are in development and clinical trials to counteract tumor growth and overcome immune evasion (37). Finally, proteolytic shedding of TAM ectodomains by A Disintegrin And Metalloproteinase 10/17 (ADAM10/17) releases soluble decoy receptors that retain ligand binding; elevated levels of soluble TAMs in autoimmune disease and cancer correlate with worse clinical outcomes, indicating that dysregulated cleavage can exacerbate pathology and serve as a disease biomarker (38).

2.6 TAM Receptors in RA

The TAM family plays a pivotal role in immune homeostasis and resolution of inflammation, with increasing evidence implicating them in the pathogenesis and progression of RA (39). Axl and MerTK are particularly relevant in RA synovium, where they exhibit differential expression in distinct macrophage subsets, influencing joint integrity and inflammatory tone (40). Axl-expressing C-X3-C motif chemokine receptor 1 (CX3CR1)+ macrophages form a synovial barrier disrupted during arthritis initiation, while MerTK is preferentially expressed by regulatory M2 macrophages, critical for tissue repair and efferocytosis (39). Notably, soluble forms of TAM receptors, particularly soluble Tyro3 (sTyro3) and soluble MerTK (sMer), circulate at altered levels in RA, with sTyro3 positively correlating with joint damage and disease activity, suggesting

potential biomarker and pathogenic roles (41). Beyond inflammation, TAM signaling intersects with bone remodeling, influencing osteoclast differentiation and osteoblast function, thus linking immune dysregulation with joint destruction (42). MerTK, in particular, has emerged as a critical regulator of osteoblast-driven bone formation and a therapeutic target in osteolytic disease models (43). Collectively, the dual role of TAM receptors in immune modulation and skeletal integrity positions MerTK as a central node at the interface of chronic inflammation and bone pathology in RA, warranting focused exploration in the following section.

3. Aim of the Study

The aim of this study was to evaluate the association between the MerTK gene polymorphism rs4374383 and major autoimmune diseases, with particular emphasis on rheumatoid arthritis.

4. Materials and Methods

4.1 Study Population

This prospective observational cohort study included 71 patients affected by autoimmune diseases, who were recruited as part of the RIVALSA study at the Rheumatology and Hepatology Units of "Maggiore della Carità" University Hospital in Novara (Italy). The study protocol was approved by the local Ethics Committee (CE 72/21) and conducted in accordance with the Declaration of Helsinki. Each participant provided written informed consent prior to enrollment.

The inclusion criteria were:

1. Presence of a diagnosed autoimmune condition (e.g., rheumatoid arthritis or other rheumatologic/immunologic disorders) requiring immunosuppressive therapy.

2. Age ≥ 18 years.

3. Clinical stability allowing participation in the study protocol.

The exclusion criteria included any psychological, social, or geographical factors that could interfere with adherence to study procedures and follow-up compliance.

4.2 DNA Extraction

Genomic DNA was extracted from whole blood samples using the NucleoSpin® Blood Kit (Macherey-Nagel, Germany), following the manufacturer's protocol. Briefly, 200 µL of whole blood was mixed with 25 µL of Proteinase K and 200 µL of Buffer B3, and incubated at 70°C for 10–15 minutes to ensure complete lysis.

Following lysis, 210 µL of absolute ethanol (96–100%) was added, and the mixture was vortexed thoroughly. The lysate was then transferred to a NucleoSpin® Blood Column and centrifuged at 11,000 × g for 1 minute to allow DNA binding.

The washing steps included:

- A first wash with 500 µL of Buffer BW, followed by centrifugation at 11,000 × g for 1 minute.

- A second wash with 500 µL of Buffer B5, followed by centrifugation at 11,000 × g for 1 minute.

To dry the silica membrane, the column was centrifuged again for 1 minute at 11,000 × g.

For DNA elution, 100 µL of preheated (70°C) Elution Buffer (Buffer BE) was added directly to the membrane. After a final centrifugation at 11,000 × g for 1 minute, the eluted genomic DNA was collected and stored at –20°C until further use.

4.3 Restriction Fragment Length Polymorphism (RFLP-PCR) for MerTK

Polymerase Chain Reaction (PCR) was performed to amplify the MerTK gene fragment containing the SNP (rs4374383) . The sequences of the primers were:

Forward: 5' AGTGATCAGGACAGGAGAG 3'
Reverse: 5' CTTTGGAAGAGTCTCGGTG 3'

The PCR reaction mix for 10 samples is detailed in Table 1. Each reaction contained 1 µL of DNA added to 9 µL of Master-Mix.

Table 1: PCR reaction components for MerTK amplification.

Reagents	**Quantity for 10 samples**
Sterile water	54.5 µL
Buffer green	20 µL
$MgCl_2$	8 µL
MerTK primer	5 µL
dNTPs	2 µL
Taq pol	0.5 µL

The PCR protocol consists in:

1. Initial denaturation: 94°C for 4 minutes.
2. 35 cycles of:
    a. Denaturation: 94°C for 30 seconds.

b. Annealing: 55°C for 30 seconds.
c. Extension: 72°C for 30 seconds.

3. Final extension: 72°C for 7 minutes.

At the conclusion of the reaction, electrophoresis of PCR products was performed on a 2.5% agarose gel to verify the amplified fragments.

Later, 3 µL of MerTK PCR product were digested with ScaI enzyme. The digestion mix is presented in **Table 2.**

Table 2 : Digestion reaction components for MerTK.

Reagent	**Volume for 1 sample**
Sterile water	5.7 µL
10X Buffer green	1 µL
ScaI	0.3 µL

Samples were incubated at 37°C for 3 hours, followed by 3% agarose gel electrophoresis using a 50 bp DNA ladder for fragment estimation.

For a Homozygous wild-type (G/G) sample, the restriction site for the Sca1 enzyme is absent on both alleles, resulting in a single visible band (211 bp). In a Homozygous mutated (A/A) sample, the enzyme cuts both alleles, producing two fragments (131 bp and 80 bp). For a Heterozygous (G/A) sample, three bands are observed (211 bp, 131 bp, and 80 bp).

Each sample underwent two rounds of PCR and digestion, and discordant results were repeated for a third round.

4.4 Statistical Analysis

Statistical analyses were conducted using Stata (version 18.0, StataCorp LLC, College Station, TX, USA). Continuous variables are described using median and interquartile range [IQR], while categorical variables are reported as frequencies (%). Genotypic frequencies were calculated for MerTK polymorphism and population was tested for adherence to Hardy Weinberg equilibrium. Association between categorical variables were analysed using Pearson Chi-square test. A two-sided p-value < 0.05 was deemed statistically significant.

5. Results

5.1 Baseline features of the study population

We recruited 71 patients affected by autoimmune diseases, of whom 36 (50.7%) were diagnosed with rheumatoid arthritis (RA). The cohort included 52 females (73.2%) with a median age of 57 years [49–68]. The distribution of autoimmune diagnoses and major comorbidities is shown in Figure 3. Autoimmune hepatitis (AIH) represented the most frequent condition (38.0%), followed by rheumatoid arthritis (50.7%), interstitial pneumonia (IPA) (31.0%), thyroiditis (14.1%). Type 2 diabetes mellitus (DM II) was present as a comorbidity in 14.1% of patients.

Other autoimmune diseases, including systemic sclerosis (SSc), systemic lupus erythematosus (SLE), Sjögren's syndrome, polymyalgia rheumatica (PMR), and psoriatic arthritis (PsA), were observed at lower frequencies (each 2.8%). Overall, RA represented the most prevalent rheumatologic condition within the cohort, while AIH was the most frequent extra-articular autoimmune manifestation.

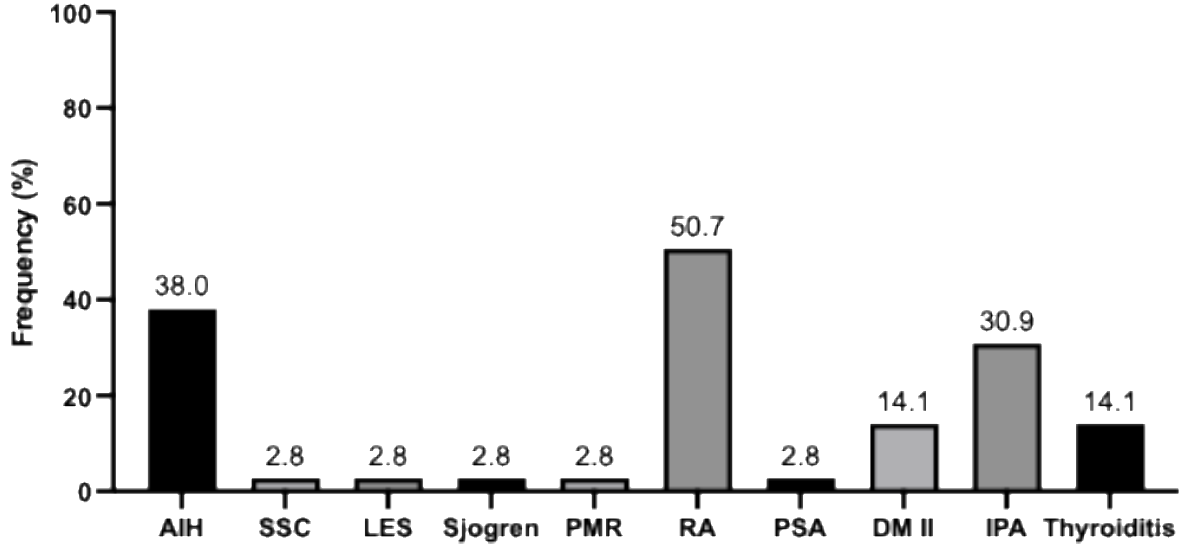

Figure *3*. Distribution of autoimmune diagnoses and major comorbidities in the study cohort (n = 71), expressed as a percentage. Abbreviations: AIH, autoimmune hepatitis; SSc, systemic sclerosis; SLE, systemic lupus erythematosus; PMR, polymyalgia rheumatica; PsA, psoriatic arthritis; DM II, type 2 diabetes mellitus; IPA, interstitial pneumonia; RA, rheumatoid arthritis.

5.2 MerTK genotype distribution

Genotyping of the MerTK polymorphism was successfully performed in all patients included in the study cohort (n = 71). The distribution of genotypes was consistent with Hardy–Weinberg equilibrium (p = 0.437). The most frequent genotype was heterozygous (G/A), observed in 49.3% of patients, while homozygous wild-type (G/G) accounted for 33.8% and homozygous mutant (A/A) for 16.9% of the whole population, as shown in the Figure 4. This pattern indicates that nearly half of the cohort carried the heterozygous form, whereas complete substitution of both alleles was relatively uncommon.

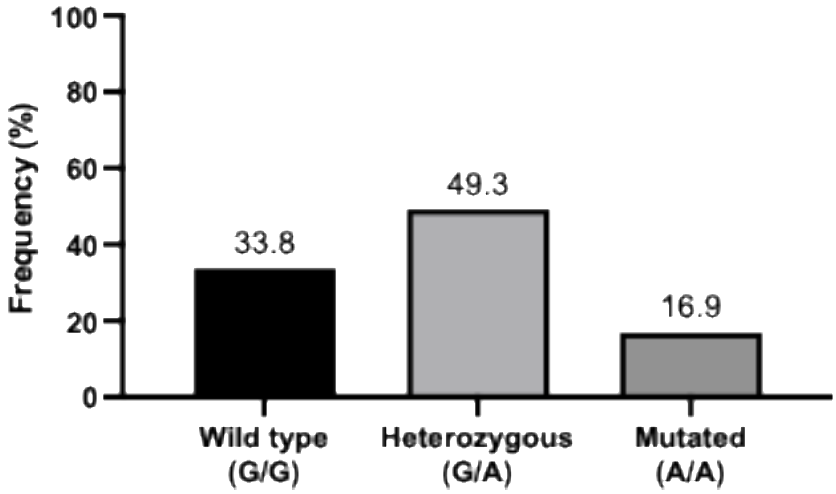

Figure 4. Distribution of MerTK genotypes in the study cohort (n = 71), expressed as percentage.

5.3 Association between MerTK polymorphism and RA

We analyzed the frequency of the MerTK genetic polymorphism as presented in Figure 5. The most frequent genotype observed in our RA patients was heterozygous, accounting for 55.6%, followed by wild type (33.3%) and homozygous mutated (11.1%). However, neither of the MerTK polymorphisms was statistically associated with RA in the cohort analyzed ($\chi^2 = 2.03$, $p = 0.362$).

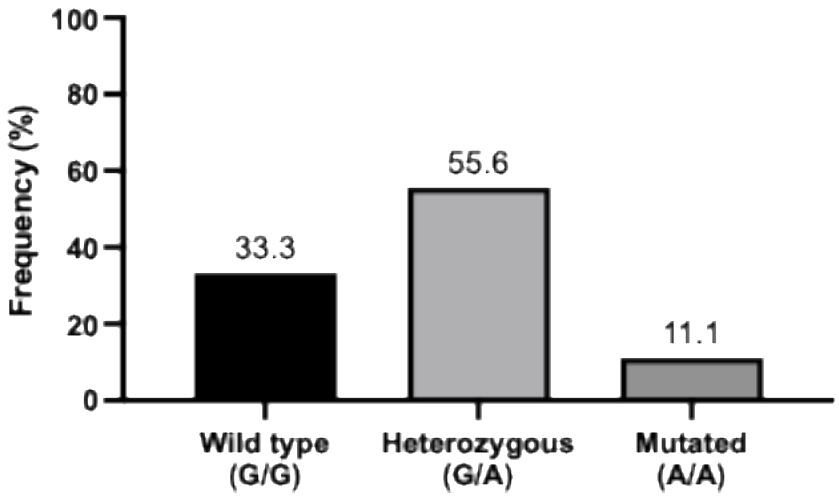

Figure 5. Distribution of MerTK genotypes in the study population. Genotype frequencies are expressed as percentages for wild type (G/G), heterozygous (G/A), and mutated (A/A) variants in the overall cohort (n = 71).

5.4 Logistic Regression Analysis

To evaluate the association between the MerTK polymorphism and the risk of developing the most frequent comorbidities (AIH, RA, IPA, DM-II and thyroiditis), a series of simple logistic regression models were performed, as shown in Figure 6. MerTK variant was not significantly associated with AIH (OR = 0.947, p = 0.877), RA (OR = 0.797, p = 0.512), IPA (OR = 0.726, p = 0.399), DM II (OR = 1.181, p = 0.734) or thyroiditis (OR = 0.927, p = 0.879). Overall, these results suggest the MerTK polymorphism is not a significant predictor of these specific conditions within our cohort.

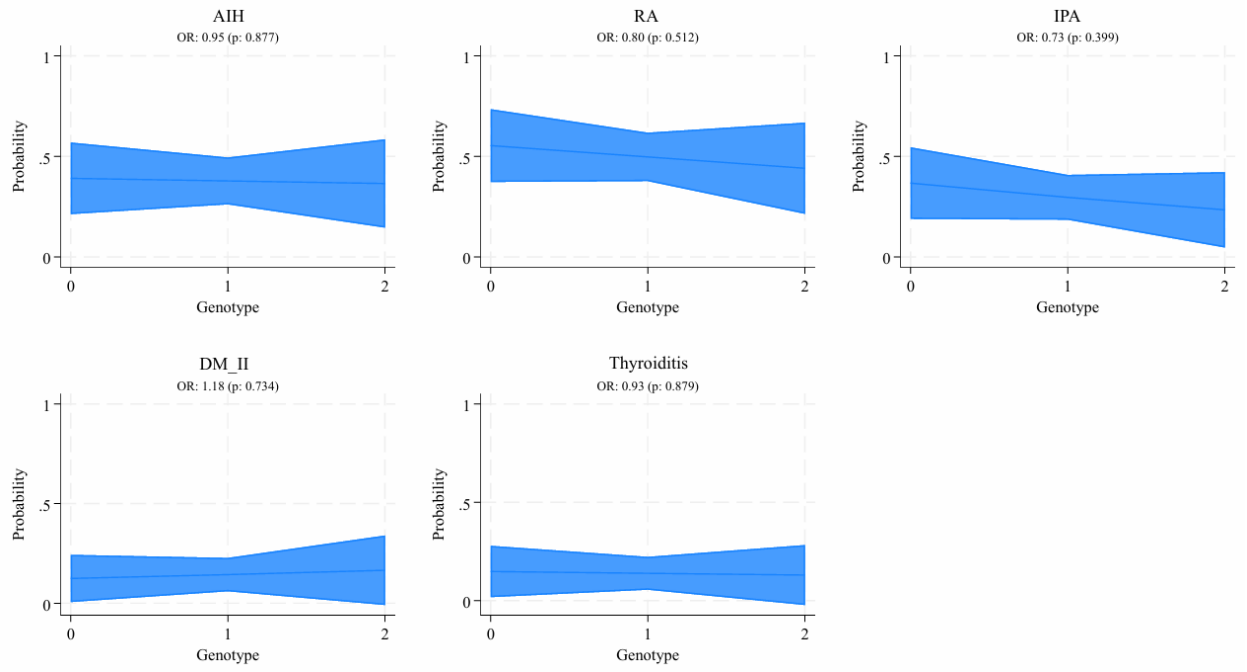

Figure 6. Predicted probabilities of disease clinical outcomes by MerTK genotype. Each panel represents a different pathology. Solid lines represent the predicted probabilities derived from simple logistic regression models, while the shaded areas denote the 95% confidence intervals (95% CI). Odds Ratios (OR) and p-values are reported for each model. Genotypes are coded as 0 (wild-type G/G), 1 (heterozygous G/A), and 2 (homozygous mutant A/A).

6. Discussion

Autoimmune diseases represent a heterogeneous group of disorders characterized by immune dysregulation, loss of self-tolerance, and chronic inflammation. Genetic susceptibility plays a central role in their pathogenesis, interacting with environmental and immunological factors. Among these conditions, RA is one of the most prevalent systemic autoimmune diseases. In this framework, we evaluated the clinical relevance of a MerTK gene polymorphism in a cohort of patients with autoimmune diseases, with particular focus on RA.

RA is a complex autoimmune disease influenced by genetic predisposition and dysregulated immune responses within the synovial microenvironment (5,11). This study investigated the distribution and clinical association of a MerTK gene polymorphism in a cohort of 71 patients with autoimmune diseases, focusing on RA. Genotyping was successfully performed in all patients and the genotype frequencies matched Hardy–Weinberg equilibrium. The most common genotype was heterozygous (G/A) followed by wild-type (G/G) and homozygous mutant (A/A). Importantly, there was no statistically significant link between the MerTK polymorphism and rheumatoid arthritis. Logistic regression analyses also failed to identify any significant association between the investigated MerTK polymorphism and the most prevalent clinical conditions observed in the cohort, including autoimmune hepatitis, interstitial pneumonia, type 2 diabetes mellitus, and thyroiditis.

Odd ratios were close to unity and all p-values were non-significant. These findings suggest that the investigated MerTK polymorphism is neither a major susceptibility factor for RA nor a significant contributor to the most common major diseases included in our cohort. Given the well-established polygenic architecture of RA, where multiple genetic variants interact with environmental factors to influence disease risk (2,11), it is likely that a single polymorphism has only a modest effect size, perhaps too small to be detected in a cohort of this size. Importantly, the lack of a significant germline association does not rule out a biological role for TAM signaling in rheumatoid arthritis pathophysiology. The TAM receptor family including MerTK is involved in macrophage polarization efferocytosis and synovial tissue inflammation resolution (39,44). Axl and MerTK expression is dynamically regulated by inflammatory state and synovial pathotype suggesting a context-dependent role in shaping the immune microenvironment rather than being static genetic determinants (39).

Furthermore, circulating soluble TAM receptors have been associated with disease activity and structural damage in rheumatoid arthritis (41). These findings suggest TAM biology might influence inflammatory modulation and immune-bone interactions at the functional or tissue level (42) even without a strong genetic association.

Our data suggest this specific MerTK polymorphism is common in autoimmune disease patients but is not significantly associated with rheumatoid arthritis or the most prevalent clinical conditions observed in our cohort.

Several limitations of this study should be acknowledged. First, the relatively small sample size may have limited the statistical power to detect modest genetic associations. In addition, the heterogeneous composition of the cohort, which included patients with different autoimmune diseases, may have reduced the ability to identify disease-specific effects of the investigated MerTK polymorphism. Furthermore, only a single genetic variant was evaluated, whereas autoimmune diseases are known to arise from complex interactions between multiple genetic and environmental factors. Therefore, larger studies involving more homogeneous cohorts and broader genetic analyses are warranted to further clarify the role of MerTK polymorphisms in autoimmune disease susceptibility.

Future research combining genetic analysis with functional approaches like soluble receptor quantification, synovial tissue profiling, and treatment-stratified cohorts will be crucial to understand if MerTK variation contributes to disease heterogeneity via context-dependent mechanisms.

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