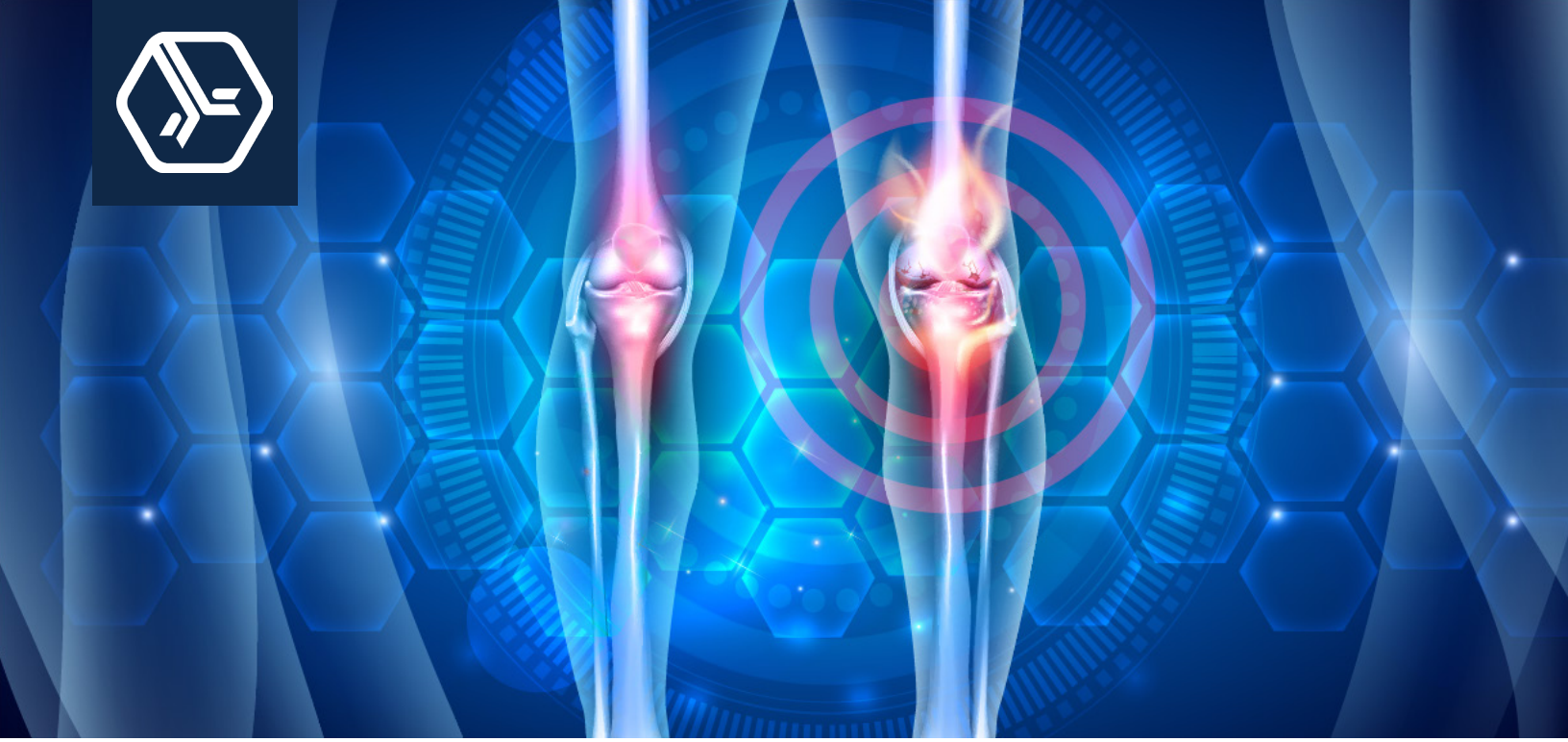

WHITE PAPER
CITRULLINATION IN DISEASE

Citrullination in Disease: Emerging Insights and Implications

- Discover the potential of citrullinated antigens as biomarkers for disease detection and patient stratification
- Learn how protein arrays differentiate autoantibody binding to citrullinated proteins in health and disease
- Explore the wide-reaching implications of citrullination in chronic inflammation and immune response

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Introduction

The dynamic landscape of proteomics is profoundly influenced by post-translational modifications (PTMs), which modulate protein structure, function, stability, localization, and enzymatic activity. Among these modifications, citrullination has attracted significant research interest across various diseases due to its involvement in processes related to chronic inflammation and its diagnostic relevance in autoimmune disorders (1).

Citrullination, a specific PTM, entails the enzymatic conversion of arginine residues into citrulline by the family of peptidylarginine deiminases (PADs) (Figure 1). This process, which is still not fully understood in the context of standard cellular activities, predominantly transpires under conditions of cellular stress. These conditions are often accompanied by inflammation, autophagy, and biological processes that increase calcium levels required for citrullination such as apoptosis, necrosis, and oxidative stress.

Citrullination in the Context of Complex Diseases

Recent investigations have shed light on the extensive role of citrullination within the etiology of complex diseases and its association with the innate immune system (Table 1). In rheumatoid arthritis (RA), anti-citrullinated protein antibodies (ACPAs) are detectable years before symptoms appear, serving as diagnostic markers in 70% of cases (2-4). They also correlate with disease prognosis (1).

The elevated levels of PAD enzymes observed in various carcinomas hint at the potential diagnostic utility of citrullinated proteins in oncology. PAD4, for example, has been detected in the blood of patients with breast, lung, colon, ovarian and prostate cancers (5,6).

Additionally, links between citrullination and neurodegenerative diseases have been explored. In patients with Alzheimer's disease, researchers have discovered citrullinated beta-amyloid protein in the brain (7). A meta-analysis of blood metabolites from dementia patients showed a significant increase in citrulline levels (8). Additionally, a recent structural analysis identified a potential citrullination site on an arginine residue in TDP-43 protein from patients with frontotemporal lobar degeneration (9). Proteins with disordered tertiary structures, such as arginine, are known to undergo citrullination readily (1). The presence of citrullinated proteins in neurological conditions opens avenues for novel diagnostic approaches. The opportunity to develop new diagnostic methods using autoantibodies against citrullinated proteins has yet to be fully investigated.

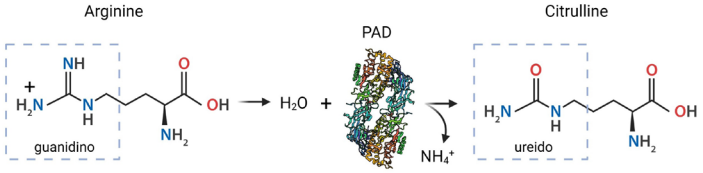

Figure 1. The citrullination process

Table 1. Proteins Commonly Citrullinated in Disease

Tissue	PAD	Proteins	Disease
Connective	PAD2, PAD4	Fibrinogen, Vimentin, Fibrin Collagen Type II, Enolase	Rheumatoid Arthritis
Tumorous	PAD2, PAD4	p53, p21, p300, ETS Like-1, Histone	Cancer
White Matter	PAD2	Myelin Basic Protein	Multiple Sclerosis
Central Nervous System	PAD2, PAD4	Vimentin, Myelin Basic Protein, Glial Fibrillary Acidic Protein	Alzheimer's Disease
Skin	PAD1, PAD3	Filaggrin	Psoriasis
Eye	PAD2	Myelin Basic Protein	Glaucoma

Challenges and Advances in Citrullination Research

The study of citrullination, characterized by its low abundance as a PTM, necessitates highly sensitive detection techniques. While traditional methods like ELISA and western blotting offer insights into the distribution of citrullinated proteins and PADs, they are limited by low throughput and semi-quantitative analysis.

Mass spectrometry (MS) and protein microarrays have emerged as powerful tools for the high-throughput, sensitive profiling of citrullination, each presenting distinct advantages and limitations in the context of sample processing and analytical specificity.

MS offers diverse methodologies to detect citrullination, tailored to specific sample requirements and investigative goals. Predominantly, a bottom-up proteomics approach using tandem MS is utilized for the direct identification of citrullinated proteins.

While a very sensitive approach, MS demands skilled technicians, involves multiple procedural steps susceptible to errors, and necessitates the use of expensive high-resolution mass spectrometers (10,11). A notable challenge with MS is that it is difficult to distinguish between citrullination and the deamidation of glutamine or asparagine.

Protein arrays present an alternative for the high-throughput and sensitive detection of citrullination, employed via direct or indirect methods. The direct method involves the application of labeled PAD enzymes to an array, enabling the screening of hundreds to thousands of immobilized proteins to identify those undergoing citrullination. This approach has led to the discovery of several new citrullinated protein substrates, predominantly involved in glycolysis, although these findings have yet to be directly linked to disease-specific citrullination (12).

Indirectly, protein arrays are used to detect autoantibodies against citrullinated proteins. This method has broad applications, including early disease detection, disease subtyping, patient stratification, and the identification of new therapeutic targets and pathways. It also provides insights into the immune system's response to this PTM.

As an example of studying citrullination using the indirect approach, sera from both anti-cyclic citrullinated peptide (CCP) positive and negative patients with diverse pathologies were analyzed using a high-density protein array. Researchers identified 844 autoantibodies, many previously undiscovered, differentiating between patient groups. This indicates potential for further stratification of RA patients and suggests that anti-CCP negative patients might be incorrectly diagnosed through conventional methods (13).

The analysis of autoantibody biomarkers via protein arrays offers significant advantages in studying citrullination's role in disease. Antibodies, typically analyzed in serum, represent a complete spectrum of circulating antibodies, are stable, and can be present long before disease symptoms manifest. This technique requires minimal sample preparation and training. Additionally, analyzing multiple antibody isotypes concurrently (e.g., IgG and IgA) can provide comprehensive information on the immune response, enhancing the accuracy of disease detection and monitoring treatment efficacy (14).

Critical Considerations in Protein Array Selection

For researchers delving into citrullination, the choice of protein array is pivotal for generating precise data. Sengenics stands out in this domain, offering a unique citrullination assay, designed to study ACPAs, with our functional protein arrays.

Utilizing our patented KREX® technology, proteins on our arrays are full length and correctly folded, crucial for the preservation of three-dimensional epitopes vital for specific antibody interactions (13, 15) (Figure 2). This precision contrasts sharply with other arrays that may rely on denatured or misfolded proteins, leading to nonspecific antibody binding and unreliable results.

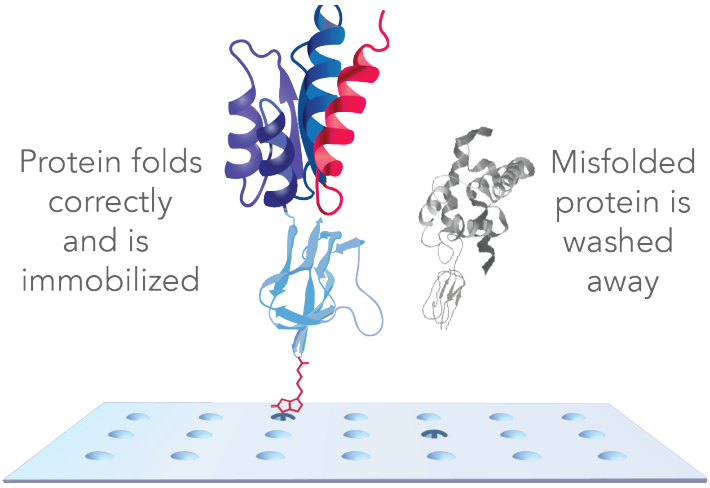

Figure 1. KREX technology for precise antibody profiling. Every protein has a tag that binds to the array surface only when the protein is correctly folded. Misfolded proteins are washed away from the array surface and removed from further analysis.

Implications for Disease Diagnosis and Therapeutic Development

Citrullinated antigens are increasingly recognized for their potential in therapeutic applications. The specificity of citrullination to diseased and autophagic tissues makes citrullinated proteins attractive targets for therapies aimed at reducing inflammation while preserving healthy cells. This approach is particularly relevant for conditions like cancer and RA.

For instance, vimentin, which becomes highly citrullinated in metastatic epithelial tumors but remains unmodified in normal tissue, has been explored as a therapeutic target. Studies in mice with melanoma have demonstrated that immunization with citrullinated vimentin significantly enhances survival rates compared to controls receiving placebo. Importantly, this strategy inflicts minimal damage on healthy tissues, underscoring the precision of targeting citrullinated proteins for disease treatment (16).

Sonoma Biotherapeutics recently shared promising preclinical findings for SBT-77-7101, a chimeric antigen receptor (CAR) T-cell therapy designed to identify and target citrullinated proteins in patients with RA, aiming to alleviate pain and inflammation. These preclinical studies successfully demonstrated the CAR T-cells' ability to recognize citrullinated proteins, directly addressing the inflammation at its source. With clinical trials slated to start in early 2024 (17), this innovative approach highlights the potential of using CAR T-cells against citrullinated proteins as a more targeted and potentially less side-effect-prone treatment compared to existing monoclonal antibody and CAR T-cell therapies. This strategy opens up new possibilities for creating highly specific treatments by leveraging the identification of citrullinated proteins.

Conclusion

The exploration of citrullination has unveiled its close association with a broad spectrum of diseases, particularly in conditions characterized by chronic inflammation and immune dysregulation. The sensitivity of current methodologies, including mass spectrometry and protein microarrays, has significantly advanced our ability to detect and analyze citrullinated proteins, thereby enhancing our understanding of their role in diseases such as rheumatoid arthritis, cancer, and neurodegenerative disorders. The potential diagnostic and therapeutic applications arising from this research highlight a promising approach for the development of targeted treatments and early detec-

tion strategies. Moreover, the specificity of citrullination to diseased tissues offers a unique biomarker for distinguishing diseased from healthy states, promising more precise and less invasive diagnostic tools.

Contact us

Sengenics develops key solutions for biological research, specializing in the discovery and validation of autoantibody biomarker signatures. For more information on how Sengenics can advance your research, email us at enquiries@sengenics.com or visit our website at sengenics.com.

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enquiries@sengenics.com | www.sengenics.com