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Recent advances in characterization of citrullination and its implication in human disease research: from method development to network integration

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Abstract

Post-translational modifications (PTM) of proteins increase the functional diversity of the proteome and have been implicated in the pathogenesis of numerous diseases. The most widely understood modifications include phosphorylation, methylation, acetylation, O-linked/N-linked glycosylation, and ubiquitination, all of which have been extensively studied and documented. Citrullination is a historically less explored, yet increasingly studied, protein PTM which has profound effects on protein conformation and protein-protein interactions. Dysregulation of protein citrullination has been associated with disease development and progression. Identification and characterization of citrullinated proteins is highly challenging, complicated by the low cellular abundance of citrullinated proteins, making it difficult to identify and quantify the extent of citrullination in samples, coupled with challenges associated with development of mass spectrometry (MS)-based methods, as the corresponding mass shift is relatively small, +0.984 Da, and identical to the mass shift of deamidation. The focus of this review is to discuss recent advancements of citrullination-specific MS approaches and integration of the potential methodology for improved citrullination identification and characterization. In addition, the association of citrullination in disease networks is also highlighted.

Keywords

Citrullination; mass spectrometry; PTM; methodology; quantitation; proteomics; disease network

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Supplemental Tables S1 and S2.

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1 Introduction

Citrullination is a post-translational modification (PTM) of great interest, characterized by the conversion of an arginine residue to a citrulline residue. Mechanistically this modification occurs through the transformation of a guanidium group, located on an arginine residue, to an ureido group, facilitated by peptidylarginine deiminase (PAD) enzymes [1–4]. In humans, PADs are a family of calcium-dependent enzymes composed of five isozymes (PAD1–4, and PAD6) which are encoded by five genes arrayed in tandem on chromosome 1 (Figure 1). These enzymes have been noted to be highly homologous, sharing 50% or higher sequence identity, with the greatest regions of similarity found toward the C-termini of the protein [4]. PADs are widely distributed in various tissues and each PAD has its specific location and preferred substrates. PAD1 is known to be primarily expressed in the epidermis and uterus, with keratins and filaggrins serving as the primary citrullination substrates within the epidermis [5,6]. PAD2 is most frequently associated with the secretory glands, uterus, spleen, and pancreas, though it is especially abundant in skeletal muscle and the brain [7–10]. PAD3 is primarily localized in hair follicles and the epidermis, where colocalization and citrullination of trichohyalin, a structural protein of the inner root sheath of hair follicles, occurs during routine hair follicle hardening [11,12]. PAD4 is found mainly in white blood cells and tumors, broadly distributed and associated with various tissue origins [13–15], PAD4 is the only isozyme with confirmed presence within the cellular nucleus, where it plays a role in histone deimination, though recent studies have reported that PAD2 may also exist in the nucleus [7]. PAD6 is essential for oocyte cytoskeletal sheet formation and female fertility, and is mainly found in mouse eggs, embryos, and oocytes [16–18]. Different from PADs 1−4, which are catalytically active, PAD6 has lost some conserved Ca^{2+} binding residues and conserved cysteine residues, thus it cannot be considered as an active deiminase [19]. A comprehensive summary of distribution of each type of PAD is illustrated in Figure 1. PAD-knockout models have served as a valuable resource to gain an understanding of the PAD-mediated roles of citrullination in various pathological pathways and diseases. In one instance, a PAD2-knockout model displayed improved survival in instances of hemorrhagic shock, a form of hypovolemic shock in which severe blood loss leads to inadequate oxygen delivery[20]. In another study, a PAD2-knockout mouse had reportedly lower levels of citrullination in the central nervous system (CNS)[21]. It has also been reported in one instance that the knockout of PAD2 did not produce any significant phenotype in the nervous system, bolstering the importance of myelin basic protein (MBP) citrullination in axonal electrical signal transmission[13], and PAD2-catalyzed citrullination was deemed non-essential for the development of autoimmune encephalomyelitis, a condition associated with inflammation[21]. Alternatively, it was determined that PAD4-knockout mice are more susceptible to bacterial infections than wild-type mice, due to a lack of neutrophil extracellular trap (NETs) formation after stimulation with chemokines or incubation with bacteria[22]. Evidence of decreased severity of autoimmune arthritis was also observed in PAD4-knockout mice[23]. Under normal physiological conditions, PADs usually maintain an inactive status, given the low internal calcium concentration. PADs can be activated and have normal functionality during certain events, such as apoptosis and terminal differentiation of cells, where calcium levels are above the physiological concentration (10^{-8} to 10^{-6} M)[24]. Given the widespread distribution of PADs, citrullination has been also reported to play

other important roles as part of normal physiological functions, including normal function of the immune system, gene regulation, apoptosis, skin keratinization, hair growth, myelin formation, NETs formation, the insulation of neurons, and the plasticity of the central nervous system[25].

The chemical mechanism of the citrullination reaction is characterized by the primary, positively-charged ketimine group of arginine undergoing replacement by a ketone group, forming neutrally-charged citrulline, a process mediated by PAD catalysis. The loss of a net charge directly impedes hydrogen bond formation and alters charge distribution, increasing the hydrophobicity of the protein and adversely affecting the protein stability (Figure 2) [26–28]. Therefore it is no surprise that many studies have defined connections between ubiquitous PAD expression, citrullination, and numerous diseases, thought to aid in the development and progression of rheumatoid arthritis (RA) [29,30], prion disease [31], psoriasis [32], Alzheimer's disease (AD)[33–35], multiple sclerosis [36–39], various cancers [40], diabetes [41,42], and others [43]. While these connections between citrullination and disease conditions are intriguing, it should be noted that a major barrier to draw definitive conclusions is presented by the scale of the existing studies, the majority of which examined a limited number of specimens, ultimately identifying only a few citrullinated proteins without site-specific localization information.

Antibody-based methods are currently the most popular, as these are well-established and documented, for detecting citrullination, though these methods are intrinsically incapable of large-scale analysis, and it is difficult to pinpoint the specific modification site [44–46]. Mass spectrometry (MS) presents a promising approach for citrullination site localization due to its excellent sensitivity, but even MS-based approaches for citrullination investigation are in dire need of optimization, as the small corresponding mass shift and low abundance of citrullinated proteins introduces significant obstacles [45,47]. Tremendous efforts have been made to overcome these challenges, working to optimize both data acquisition and data analysis processes. With respect to acquisition method optimization, methods for chemical derivatization [48,49] and enrichment [50–52] have been presented, while others have worked to generate novel algorithms for data analysis and database searching [53–58], in addition to approaches complemented with careful, manual examination of spectra [59,60]. These methods seek to overcome the intrinsic hurdles encountered with citrullination identification, including its small mass shift, by inducing a larger mass shift for citrullinated peptides compared to their non-modified counterparts for more confident identification; low abundance, by effective enrichment methods; and localization, by developing algorithms to increase statistical confidence of MS identification results. The aim of this review is to introduce and survey novel MS-based citrullination identification approaches and investigate the ways in which these methods can be integrated into citrullination investigations, motivated by network biology. The involvements of citrullination in the pathogenesis of various diseases are also discussed. A comprehensive overview of citrullination-associated diseases along with corresponding tissues, proteins, cell types, and PAD isozyme types is presented (Supplementary Table 1), as well as the utilization of MS techniques and methodologies that lead to these biological findings (Supplementary Table 2).

2 Strategies to identify citrullination sites and method integration

2.1 Antibody-based and probe-based detection of protein citrullination

Our knowledge of the citrullinome is still limited primarily due to the lack of effective and robust analytical tools, as many rely on low-throughput methods, such as immunodetection using available antibodies that recognize citrulline residues, identified through traditional Western blot or immunostaining procedures [46,61,62]. Two primary polyclonal antibodies are used for citrullination detection, histone H3, which recognizes citrulline residues at positions 2, 8, and 17, and histone H4, which recognizes these residues at position 3. Anti-peptidyl-citrulline, clone F95 antibody has also been validated for detection of peptidyl-citrulline [63,64]. Clonal F95 antibodies have been employed to recognize citrulline by staining the fibrinoid extracellular matrix of necrotic synovial tissue of RA patients, enabling visualization of citrullinated proteins in those areas. Another antibody-based method utilizes further modification of any present citrulline residues with 2, 3-butanedione monoxime and antipyrine in a strong acidic solution, which forms an ureido group adduct, detectable by Western blot with an anti-modified citrulline antibody [65]. Moreover, a citrulline-specific probe, rhodamine, is a sensitive fluorophore that specifically detects protein citrullination via a chemoselective reaction between the glyoxal group of the rhodamine and citrulline [64,66]. Although these methods are sufficient for some applications, they lack specificity, the sensitivity of the antibodies is less than ideal, they are incapable of profiling citrullinated proteins of low abundance, and they cannot provide localization of the citrullinated residue, rendering these approaches unfit for global citrullinome characterization.

2.2 Standard Mass Spectrometry Approaches

MS-based strategies are gaining popularity as powerful tools for large-scale characterization and localization of various PTMs due to the rapid development of MS sensitivity, accuracy, and compatibility, though its application to mapping citrullination is much less established [44,45,47,67,68]. As citrullinated proteins are often present in low abundance, corresponding signals can be largely suppressed by other molecules within the sample, with effective enrichment solutions lacking. In the event that the low abundance citrullinated protein is detected, the small mass shift induced by citrullination (+0.984 Da) can be easily confused with deamidation (+0.984 Da) and ^{13}C isotopic peaks (+1.0033 Da). These hurdles result in poor-quality tandem MS spectra, presenting additional challenges in confident identification, localization, and quantification of citrullinated proteins. Standard MS analysis is possible, but often requires a mass spectrometer with high mass accuracy and time-consuming manual examination of the MS spectra [59,69,70]. Therefore, optimized strategies for citrullination sample preparation, MS parameters, and data searching have been developed to fill these gaps (Figure 3). These approaches and their effectiveness will be discussed in the rest of this section.

2.3 Fragmentation characteristics of citrullinated peptides

To combat these issues, significant effort has been devoted to leverage the characteristics of fragmentation to aid in the efficiency and accuracy of citrullination site detection and localization. An abundant neutral loss of 43 Da from citrullinated peptide precursor ions was detected by collision-induced dissociation (CID), as the HNCO moiety, isocyanic

acid, is eliminated from the citrulline ureido group. This loss was reportedly observed not only in multiple charge states of precursor ions, but also in b- and y-fragmented ions, providing a diagnostic marker for citrullination identification [71]. This observation became the foundation for confident citrullination discovery and profiling, and has been successfully adopted by numerous citrullination identification workflows and applications [51,52,72,73]. For example, Lee and colleagues made tremendous headway by mining the human tissue proteome for protein citrullination evidence from 30 human tissues by MS, using this foundational knowledge as a starting point. By combining fragmentation characteristics associated with the neutral loss of isocyanic acid with manual interpretation of the spectrum, coupled with the use of reference spectra for deiminated peptides or synthetically citrullinated peptides, database searching of 70 million tandem mass spectra yielded 13,000 candidate citrullinated spectra, which were further filtered by spectra quality metrics and the detection of the diagnostic ions from citrullinated peptides to reduce false positives. The team also synthesized about 2,200 citrullinated and 1,300 deamidated peptides to build a library of reference spectra, enabling the validation of 375 citrullination sites from 209 human proteins [59]. While this study expanded the human protein citrullination database, it also demonstrated a possible solution to increase the confidence of citrullination identification, providing a robust method that can be used to explore the biological roles of these modifications in the future. While these findings and the implementation of quality assurance methods are invaluable, it must be noted that the time devoted to the manual interpretation of the spectrum and the synthesis of thousands of peptides is simply unfeasible for global investigations where high-throughput procedures are a necessity. With this, Maurais and colleagues developed a streamlined data analysis pipeline for citrullination identification using an automated workflow to rigorously and rapidly mine proteomic datasets to identify the sites of citrullination from complex peptide mixtures via ionFinder and envoMatch software programs. Citrullination sites were classified with high confidence based on the presence of diagnostic fragment ions, specifically the neutral loss of isocyanic acid, as a marker for the diagnosis of protein citrullination. They used this method to map the sites of autocitrullination on purified PADs 1−4, as well as global citrullinome in a PAD2-overexpressing cell line, and finally used this framework to identify more than 350 unique citrullinated peptides from 220 proteins. Many of the proteins identified to be citrullinated were nuclear proteins, further supporting the nuclear localization of PAD2 [56]. With the fast search speed of automated software compared to manual inspection, this method significantly improved the efficiency and throughput of identification of citrullinated proteins. EnvoMatch and ionFinder are good complements to commercially available software for database searching, with the ability to increase the confidence of peptide-citrullination assignments. Automated annotation of diagnostic neutral loss species by standard database search algorithms is still a core process to verify the assignment of citrullination. In addition, the customizable nature of these post-processing tools has potential to benefit other proteomic analyses, by defining stringent criteria for modified peptide assignment, though it should be noted that the described automation program is not publicly available for others to consult. Despite these advancements, low-abundance proteins still pose a major challenge, as coverage of citrullination events in these scenarios is limited, generating a bias in terms of identification of citrullination sites from abundant proteins

within the proteome. Selective enrichment of citrullinated peptides is thus a necessity for comprehensive citrullination site identifications.

2.4 Citrullination data integration with database analysis and machine learning

When abundance becomes a limiting factor in the identification of PTMs, many have turned from traditional data-dependent acquisition (DDA) methods to data-independent acquisition (DIA) methods [74–76]. While DDA methods operate so that the top *n* most abundant precursor ions are selected for fragmentation, biasing against ions of low abundance, DIA methods ensure the fragmentation of all precursors, in essence minimizing this biasing issue [77,78]. To accomplish this, the precursor m/z range is essentially divided into windows, of a selected m/z width, and all precursors within this window are subjected to fragmentation [78,79]. Though attractive for these applications, DIA methods can present tremendous challenges in data analysis, specifically with deconvolution [80].

Fert-Bober and colleagues developed a citrullination-targeted proteomic strategy using a DIA method to improve quantitative consistency and accuracy of identifications [28]. This method involved the development of a citrullinated peptide spectral library, used to parse identifications from complicated DIA output spectra, followed by downstream quantification. After validation using two-dimensional gel electrophoresis, 304 citrullinated sites were identified belonging to 145 proteins in human myocardium samples [28]. While these initial results were promising, identifications were limited to peptides already present within their spectral library. Aiming to adapt their method for larger-scale investigation, the same team generated a robust mouse hyper-citrullinated spectral library. In order to draw comparisons between modified and unmodified peptides, spectral matching of both forms was accomplished through the use of delta retention time shift (ΔRT) as a signature for citrullination [53]. Subsequent validation of findings was achieved through the detection of the neutral loss of isocyanic acid in peptides within the CID spectra using Skyline software. In total, they identified 3026 citrullinated peptides in 1037 citrullinated proteins, 90% of which were novel, from several mouse organs, probing their involvement in a variety of biological processes [53]. This work provides a rich resource of candidate citrullinated proteins and potential citrullinated biomarkers in clinical research. With several cases of citrullination library utilization [56,59], machine learning (ML) emerged as an attractive route to generate models capable of predicting citrullination position. Chaerkady and team generated a random forest ML prediction model to characterize citrullination sites in neutrophils and mast cells via integration of MS and ML [81]. Prior to ML, they performed preliminary data analysis using MaxQuant software with stringent, automatic filtering of peptides, obtained from neutrophil and mast cells treated with and without ionomycin, with the expected neutral loss signature from the MS/MS output files used to ensure the presence of at least one citrullination site in candidate peptides. Preliminary data analysis yielded a total of 833 validated citrullination sites on 395 proteins. Using this benchmark dataset, they developed random forest ML-based prediction models, one for each neutrophil and mast cell analyses, for identification of citrullinated protein sites according to motif presence [81]. Of note, one-fifth of PAD4 substrates contained an RG/RGG motif, which has been reported in previous studies [82], and Gly and Asp residues were largely found surrounding citrullinated sites within the ML work [81]. This study further reveals substrate motif preferences for

PAD enzyme−substrate interactions and provides insight into citrullination of proteins in important biological processes. To date, this was the first report that integrated the use of ML in MS-based citrullination analyses and enabled the prediction of a catalytic motif for PAD2 and PAD4. These catalytic motifs need further experimental validation, which will improve the understanding of PAD catalysis as well as aid in future drug or inhibitor design.

2.5 Probe design and development for citrullination identification

As the small mass shift induced from citrullination can be easily misinterpreted as deamidation or another isotope with a similar mass, a chemical probe specifically designed to target citrullination sites would be advantageous, enabling an increase of the observed mass shift. De Ceuleneer and team developed a chemical tagging strategy, which allowed them to selectively pinpoint citrullinated peptides in a complex mixture with liquid chromatography-mass spectrometry (LC-MS) analysis [48]. Citrulline residues in the peptide mixture were able to covalently react with 2, 3-butanedione, which resulted in a 50 Da mass shift from singly charged ions. With this, citrullinated peptides in the mixture could be identified by comparison of the peptide mass fingerprint of a modified and an unmodified version of the same sample. The reaction conditions, including pH and incubation time, were then optimized, with synthetic peptides, and validated using a digest of citrullinated fibrinogen, a protein highly implicated in citrullination-mediated disease pathogenesis [83]. It should be emphasized that the use of an acidic reaction environment, specifically necessary for citrulline-containing residues, should be strictly controlled, as carrying out the reaction under neutral or basic conditions can cause arginine to adversely react. This was the first study to introduce a mass probe for easier detection of citrullinated residues and was also a milestone study, as it inspired further citrullination derivatization design [51,52,72,73].

As the abundance of citrullinated proteins is typically low in biological systems, an effective enrichment strategy is needed for MS analysis. Tutturen and team presented a technique for both specific modification and selective enrichment of citrullinated peptides from biological samples [51]. The technique relied on an induced reaction between a glyoxal derivative, 4-glyoxalbenzoic acid (GBA), and the ureido group of the citrulline residue under strong acidic conditions, with an additional biotin moiety attached to the GBA molecule, enabling further enrichment by streptavidin beads, producing biotinylated, citrullinated peptides upon biotin-PEG-GBA (BPG) modification [51]. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) was conducted to analyze the impact of the enrichment strategy, revealing that the sensitivity of citrullination detection was greatly improved, though performance of the BPG modification in LC-MS for high-throughput citrullination profiling remains unknown. Lewallen and team developed a biotin-conjugated phenylglyoxal (biotin-PG) strategy to enrich, detect, and quantify protein citrullination [52]. This probe was shown to serve as an antibody surrogate, compatible with Western blotting, for the enrichment of citrullinated proteins from complex mixtures. Moreover, the probe was implemented for MS identification, serving as an enrichment tag to identify and quantify abundance of citrullinated proteins among different biological samples. More than 50 citrullinated proteins were identified and significantly enriched by at least 2-fold in a PAD2-overexpressing cell line compared to the controls using this probe and platform technology. More recently, Shi et al. reported the design and development

of a biotin-thiol tag that enabled derivatization, enrichment, and confident identification of citrullination via MS with high specificity and efficiency [73]. They performed proof of principle experiments using recombinant human histone H3 protein with or without *in vitro* PAD. The team also explored different fragmentation techniques and enzymatic digestion methods for optimized citrullination analysis, with LysC/trypsin digestion in conjunction with stepped higher energy collision dissociation (HCD) fragmentation providing the best coverage of the citrullinome. In total, 691 citrullination sites from 432 proteins were globally mapped through large-scale citrullinome profiling of different mouse tissues [73]. This work exemplified an effective and greatly improved MS-based approach, with higher reproducibility and accuracy than previous methods. It should be mentioned that this work presented a new area in need of investigation, as fundamentally, citrulline is resistant to trypsin digestion, though their work identified peptides with C-terminal citrullination sites, which were treated as potential artificial identifications being removed from the citrullination identification list. However, these results and complementary experiments suggest that the cleavage might be protein-specific, or occur at a lower rate, which has been discussed in other studies [60,84]. More in-depth characterization might be needed to fully elucidate the underlying mechanism of the observation of C-terminal citrullination products.

Isobaric mass tagging strategies provide a unique avenue to achieve relative quantification, by enabling higher sample throughput and improved accuracy. Custom-developed N, N-dimethyl leucine (DiLeu) isobaric tags serve as a cost-effective alternative to expensive commercial isobaric tagging reagents, such as TMT, presenting a valuable tool for high-throughput MS-based quantitative proteomics [85,86]. Recent work has combined the aforementioned novel derivatization methods with DiLeu isobaric tags to achieve multiplexed quantitative analysis of citrullination from up to 12 samples [72]. This approach was applied to investigate citrullination alterations in response to DNA damage-induced stress using human cell lines. In total, they identified and quantified 78 citrullination sites from 63 citrullinated proteins in three DNA damage treatment groups and one control group [72]. This strategy combined the pipeline for isobaric labeling with previous methods for derivatization and enrichment of citrullinated peptides, enabling high-throughput quantitation of citrullinated proteins from complex biological samples with greatly improved accuracy. This approach provides a simple yet powerful tool for unambiguous, high-throughput identification and quantification of citrullinated peptides, which could be easily adopted by other researchers in the field. Clearly, the established target MS methodology coupled with a top-down proteomics approach is still needed to capture specific citrullinated proteins with unique properties, such as highly basic histone proteins [72].

Determination of sites citrullinated by PAD using ^{18}O stable isotope labeling was previously conducted via MS [87]. In this method, an oxygen atom was incorporated into the citrulline residue from H_2O during citrullination by PAD, peptides citrullinated in 50% $H_2{}^{18}O$ could be recognized through a characteristic isotope distribution which could be distinguished from its natural abundance, making the citrullinated sites easier to be detected. To demonstrate the proof of concept with the peptides citrullinated in 50% $H_2{}^{18}O$, the authors conducted *in vivo* experiments of human fibrinogen citrullination identification by PAD4 treatment. The results showed complete agreement of identified citrullinated sites and equivalent sequence coverage with MS/MS spectra. We believe this methodology can be

more readily adapted to *in vitro* protein citrullination identification and validation, though in analyses of a complex protein mixture *in vivo*, this method will still require time-consuming manual examination of the MS spectra given the small mass shift.

2.6 Top-down and bottom-up proteomics integration

With the development of advanced MS instrumentation and improved enrichment strategies, the integration of different proteomics methodologies can facilitate more comprehensive investigation into citrullination to answer specific questions. Top-down proteomics (TDP) employs MS to analyze intact proteins for effective characterization of PTMs, while bottom-up proteomics (BUP) examines digested protein-derived peptide sequences, localizing PTMs using tandem MS analyses. TDP enables the identification of novel protein isoforms and PTMs, characterization of sequence variations, and quantification of pathological alterations in a form highly similar to the protein's native form [88]. However, few studies have explored citrullination profiles achieved with TDP, as these methods are considerably more challenging to analyze compared to BUP approaches, particularly due to the complexity of the generated data and instrumental limitations [89]. One TDP study presented an electrospray ionization (ESI) MS method to observe the structural alteration of recombinant human histone H2A/H2B dimers induced by PAD4 citrullination, finding that citrullination stabilizes the histone dimer association [90]. Citrullination-related PTM crosstalk is another important but complex biological event in need of more attention, as it influences complex phenotypic outcomes, thus inducing physiological and pathological alterations. The existence of crosstalk between citrullination of H3R26 and methylation of H3K27 has been reported [91]. We believe the combination of complementary information from BUP and TDP analyses will be beneficial to capture more crosstalk information.

2.7 Site-specific incorporation of citrulline into proteins

Despite recent advances for identifying citrullination sites, it still remains challenging to investigate the functional impact of citrullination with respect to a single residue. Mondal et al. recently reported a technology that enables the site-specific incorporation of citrulline into proteins in mammalian cells using an *Escherichia coli*-derived engineered leucyl tRNA synthetase-tRNA pair[92]. This approach incorporated a photocaged-citrulline (SM60) into proteins in response to a nonsense codon. SM60 can be further converted to citrulline with light *in vitro* and in living cells. To demonstrate proof of concept, they further characterized the effect of incorporating citrullination at two known auto-citrullination sites in PAD4 (R372 and R374) and showed that these two mutants were 181- and 9-fold less active than the wild-type enzyme, respectively. This work elucidated how these modifications could impact enzyme activity and indicated potential to decipher the biology of citrullination with this technology. This strategy enables the incorporation of citrullination on demand and mechanistically addresses how this PTM impacts fundamental biological processes and pathways, which will ultimately pave way for further functional validation of citrullination coupled with traditional knockout or knockdown experiments.

3 Networks of citrullinated proteins implicated in various disease phenotypes

With a growing number of studies implicating citrullination in disease pathology, this modification is now considered to be a hallmark of disease progression in many scenarios. In this section, we summarize the reported citrullinated proteins and corresponding autoantibodies that are responsible for disease pathogenesis and development including rheumatoid arthritis, neurodegenerative diseases, cancer, and diabetes (Figure 4). These relationships highlight the citrullinated hallmarks that may serve as probable targets for diagnosis and treatment solutions.

3.1 Rheumatoid Arthritis (RA)

RA is a chronic, progressive autoimmune disease that primarily affects the lining of synovial joints and can lead to pain, functional disability, and premature death [93]. Citrullination is considered to be a major contributor to RA disease pathogenesis, with citrullinated proteins serving as major targets of antibodies in patients with RA. The participation of citrullination in RA pathogenesis has been observed in several contexts. Dysregulated PAD activity and aberrant expression of citrullinated proteins were found in the synovial fluid of RA patients, including citrullinated trichohyalin, filaggrin, histones, and transcription factors [29,30]. Furthermore, anti-citrullinated protein antibodies (ACPAs) have been found to be produced against various citrullinated protein antigens and have frequently been found in many affected individuals, with the presence of ACPAs in RA linked closely with disease severity [29,52,72,81,94]. With this, generation of ACPAs at early developmental stages can have a strong predictive value and be used for early RA diagnosis. The development of ACPAs is also considered critical for RA pathogenesis, with observation of autoantigens for ACPAs in the synovial fluid, including filaggrin, fibronectin, fibrinogen α- and β-chains, α-enolase, vimentin, type II collagen, immunoglobulin binding protein-BIP, tenascin-C, as well as microbial components VCP1 and VCP2 [95–99]. Another autoantibody discovered in RA patients is the rheumatoid factor (RF), which was determined to be directed against serum γ-globulins and have a strong association with RA [100]. RF has been found in multiple immunoglobulin isotypes, including IgM, IgG, and IgA; RF is capable of directly binding to the Fc portion of aggregated globulins. RF has been used as a biomarker for RA testing, with reported 60% to 90% sensitivity in patient blood samples [101]. PAD2 and PAD4 are the two main isozymes determined to mediate citrullination in RA, both of which have been localized in neutrophils [102]. Inflammation is one of the trademark characteristics of RA and results from atypical citrullination, which promotes the formation of NETs, a network of extracellular strings of DNA that bind pathogenic microbes [103,104]. It has also been found that citrullinated histones from neutrophils were routinely targeted by ACPAs, suggesting that increased histone citrullination could be linked to increased autoimmune response [105]. Several other citrullinated proteins reported to be in NETs include actin, actin related protein 2/3 complex subunit 1B, coronin, and leukocyte elastase inhibitor [106,107].

3.2 Neurodegenerative diseases

Alzheimer's disease (AD) is the most common neurodegenerative disease (ND), characterized by the abnormal aggregation of misfolded proteins, such as amyloid beta (Aβ) and phosphorylated tau protein within the brain [108,109]. To date, the development of therapeutic approaches targeting these two biomarkers has been lacking. It has been suggested that excessive expression of PAD isozymes, specifically PAD2 and PAD4, with the PAD2 isoform considered to be predominantly expressed in the CNS while PAD4 has been observed in the hippocampus and cerebral cortex, can contribute to ND changes implicated in AD pathology [33,110,111]. Another study found an abnormal accumulation of citrullinated proteins and an increase of the PAD2 content in the hippocampus of AD patients [33]. Most recently, citrullination of Aβ in AD brains was detected by MS-based methods, concluding that a common mechanism for citrullination of Aβ exists in both the sporadic and familial representation of AD, and that citrullinated Aβ peptides could possibly trigger adverse immune response [112]. The most frequently reported citrullinated proteins associated with AD are often structural proteins, such as fibrillary acidic protein (GFAP), myelin basic protein (MBP), vimentin, and neurogranin (NRGN), with 12 citrullination sites on MBP, 2 citrullinated sites on vimentin, 4 citrullinated sites on GFAP, and 1 citrullinated site on NRGN detected by either MS-based methods or monoclonal antibody strategies [60]. GFAP, a cytoskeletal protein of astrocytes in the AD brain, is a substrate compatible with PAD2, indicating that citrullinated GFAP may function in the progression of AD [113]. The relationship between ND and citrullination is not limited to AD, as elevated citrullinated MBP levels have also been reported in multiple sclerosis patients, representing an important biochemical pathway in its pathogenesis [36]. Over 27% of MBP arginine residues are citrullinated in multiple sclerosis compared to healthy individuals [38]. Hypercitrullinated MBP has been shown to result in a partial conformational change, hypothesized to suppress the transition of lipid bilayers into compact multilayers, resulting in loss of proper nerve signal transduction [114].

3.3 Cancers

Citrullination has also been reported to participate in various processes associated with the pathogenesis of cancer and tumor biology. PAD is also an active area of cancer therapeutic and biomarker development. The pathway of this relationship is quickly becoming clearer, as new evidence shows that PAD2 and PAD4 enzymes are overexpressed in many types of neutrophils and tumor cells, such as adenocarcinoma and prostate cancer cells [102,115]. PAD4 has also been documented in association with gastric cancer, liver cancer, and ovarian cancer [116–119]. PAD4 levels were also found to be elevated in blood samples of patients with lung cancer [120]. Colorectal cancer liver metastatic growth was reported to be relevant to PAD4-driven citrullination of the extracellular matrix [14,121]. These findings suggest the important role of citrullination in metastasis and disease progression. Furthermore, PAD2 appears to play a tumorigenic role in breast cancer, digestive system cancers, and skin cancers [122]. A well-known tumor biomarker, cytokeratin, was found to be citrullinated, and as a result prevent caspase digestion, suggesting its citrullinated form may serve as a novel target for tumor treatment [120]. It was found that PAD4 and histone hypercitrullination are crucial for chromatin unfolding and the formation of NETs, as histone citrullination can not only regulate various cellular processes but also

alter the formation of NETs. In addition, citrullinated histones are typically associated with the promotion of NETs-mediated inflammation by regulating the localization and activation of toll-like receptor 4 (TLR4) [123,124]. PADs and citrullination also regulate gene transcription and induce response to DNA damage. For example, most of the p53 target genes are tumor suppressor genes which regulate cell cycle arrest, DNA repair, metabolism, translation control, programed cell death, and autophagy [125]. The level of citrullinated arginine 3 residues of histone H4 is negatively correlated with p53 protein expression and tumor size in non-small cell lung cancer tissues [126]. Citrullination of H4R3 and Lamin C in response to DNA damage was demonstrated to induce nuclear fragmentation through the p53-PAD4 pathway [127]. Citrullination and methylation crosstalk on histone H3 are also reported to regulate ER-target gene transcription. H3K27 demethylases were able to activate transcription after H3Cit26 formation, which supports the existence of crosstalk between citrullination and methylation in the adjacent position [91]. It can be hypothesized that citrullination crosstalk may have a crucial role in carcinogenesis through the effect of histone modifications on aberrant tumor suppressor genes.

3.4 Type 1 diabetes (T1D)

T1D is an insulin-dependent glucose metabolic disorder caused by inflammation of pancreatic islet cells, specified as an organ-specific autoimmune disease induced by autoimmune response against pancreatic β cells. It was first reported that insulin B can be citrullinated in *Porphyromonas gingivalis*, with subsequent publications establishing the relationship between citrullinated β-cell proteins and coordinated response in T1D [128,129]. More recently, PADs and citrullination have emerged in additional T1D-associated pathways, coupled with several key proteins responsible for blood sugar regulation, encompassing glutamic acid decarboxylase 65 (GAD65), islet amyloid polypeptide (IAPP), islet antigen-2 (IA2), glucose-regulated protein 78 (GRP78), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) [41,42]. In recent studies, citrullinated GRP78 and GAD65 were discovered as novel autoantigens when human islets were exposed to inflammatory stress induced by interleukin-1β, tumor necrosis factor-α, and interferon-γ, thus contributing to loss of immune self-tolerance toward β-cells in T1D [42]. The citrullination of glucokinase (GK) was also identified as a result of inflammation, triggering an autoimmune response. Citrullination of GK is also known to alter its biological activity and suppress glucose-stimulated insulin secretion [130]. Surmounting evidence has shown the citrullination participation of neutrophils and NETosis in the pathogenesis of T1D. Proteomic profiling has indicated that the level of PAD2 is higher in prediabetic nonobese diabetes (NOD) islets than the control group [131]. Inflammatory cytokines, which activate endoplasmic reticulum (ER) stress pathways, were shown to induce citrullination of β-cell proteins. These findings support a potential therapeutic strategy of inhibiting PAD enzymes and utilizing specific citrullinated autoantigens as drug targets for T1D.

Conclusions and future directions

This review presents a frame of reference for the role of citrullination concerning the pathogenesis of several diseases including RA, cancer, ND, and T1D. It also highlights novel MS techniques and their applications that facilitate profiling of citrullination sites

with diagnostic and therapeutic significance. The precise identification and characterization of protein citrullination remains challenging and quantification is even more convoluted due to the limited approaches available. There are many technological gaps in this area, as well as many plausible hypotheses to investigate. Future integration of these methodologies with biological networks will promise to provide a foundation for the elucidation of these underlying mechanisms and disease pathologies. Some immediate areas of examination are necessary for the field to progress, namely, there are discrepancies in the relationship between trypsin cleavage and citrullination sites, as these sites should not be cleaved, though experimental evidence has reported C-terminal citrulline residues on a number of occasions [43,54,60,73]. Most importantly, future research efforts should not only develop robust and efficient methodologies to expand the “citrullinome” database, but also explain corresponding pathways to establish viable therapeutic targets and produce novel drugs for the treatment of citrullination-associated diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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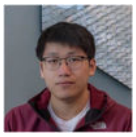
Biographies



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Abbreviations

PTM	Post-translational modifications
LC-MS	liquid chromatography-mass spectrometry
PAD	peptidylarginine deiminase
CID	collision-induced dissociation
DDA	data-dependent acquisition
DIA	data-independent acquisition
ΔRT	delta retention time shift
ML	machine learning
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
HCD	higher energy collision dissociation
RA	Rheumatoid Arthritis

ACPA	Anti-citrullinated protein antibodies
AD	Alzheimer's disease
RF	rheumatoid factor
CNS	central nervous system
T1D	Type 1 diabetes
NETs	Neutrophil extracellular traps
NOD	prediabetic nonobese diabetes
BUP	bottom-up proteomics
TDP	top-down proteomics
ESI	electrospray ionization
ER	endoplasmic reticulum
GFAP	glial fibrillary acidic protein
MBP	myelin basic protein
NRGN	neurogranin
ND	neurodegenerative disease
TLR4	toll-like receptor 4
GAD65	glutamic acid decarboxylase 65
IGRP	islet-specific glucose-6-phosphate catalytic subunit-related protein
GK	glucokinase
DiLeu	N, N-dimethyl leucine

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Significance statement

Citrullination is a key PTM that affects protein structure and functionality. It has been associated with the development of diverse pathological states and has raised much interest in recent decades, though advancement in this area has been hindered due to challenges associated with enrichment, detection, and localization of protein citrullination. This review summarizes recent advances in MS-based citrullination characterization approaches and methodology integration. The impact and association of protein citrullination on signaling networks in disease pathogenesis and progression is also highlighted in this review.

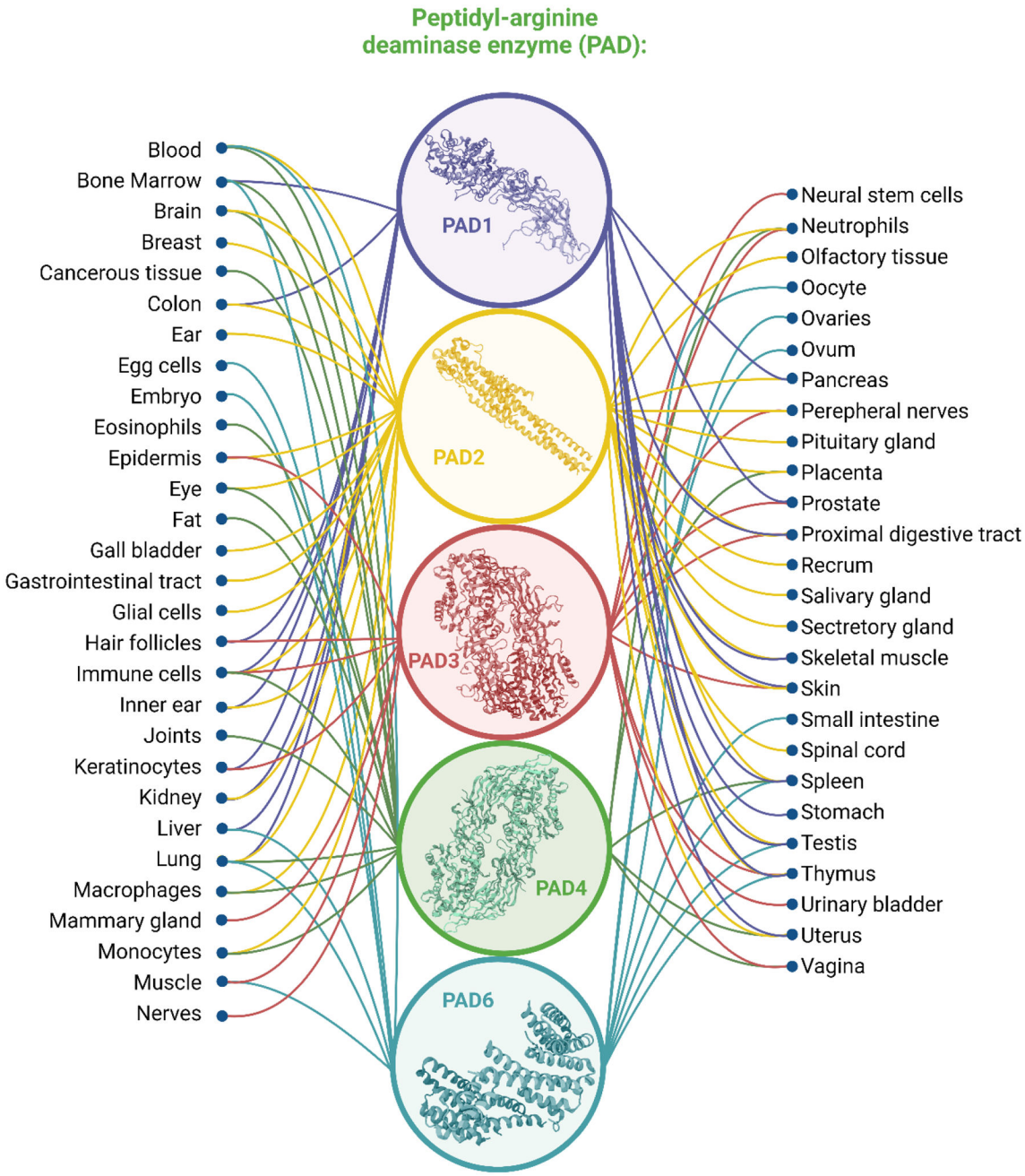

Figure 1.
Five types of peptidylarginine deiminases (PADs) and their corresponding distribution.

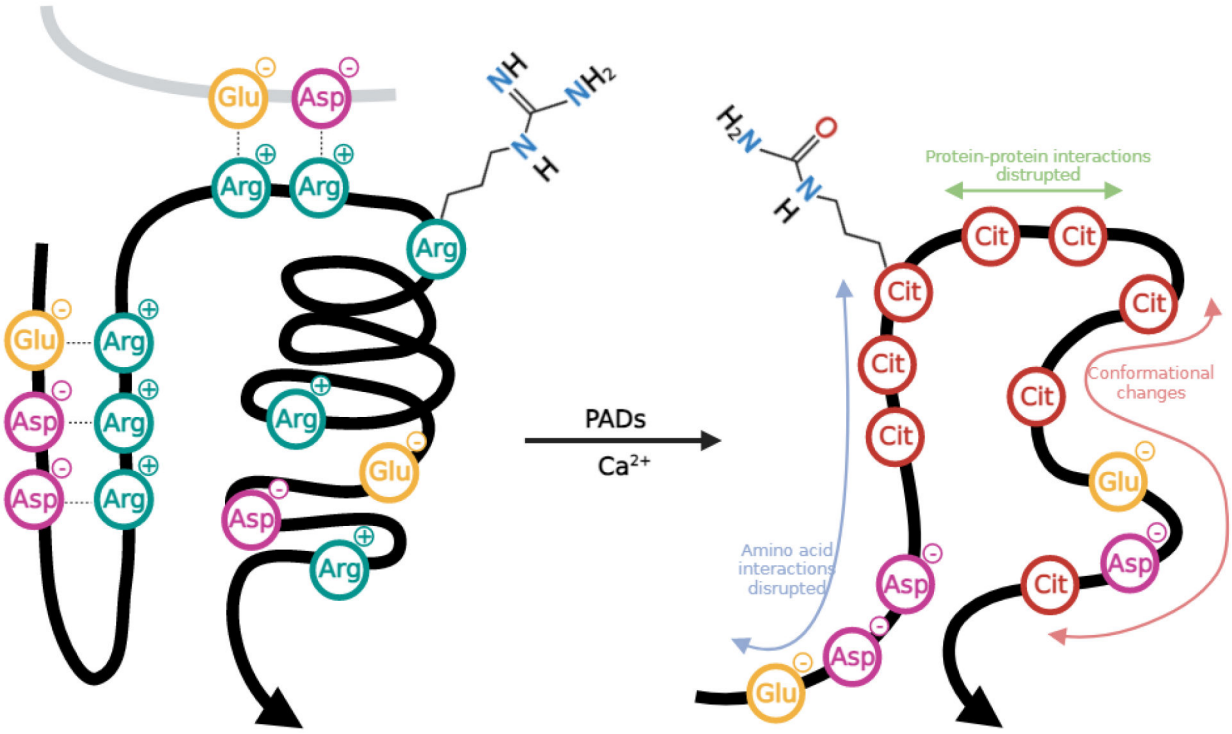

Figure 2.

Citrullination of arginine by peptidylarginine deiminases and the subsequent impact on protein–protein interactions.

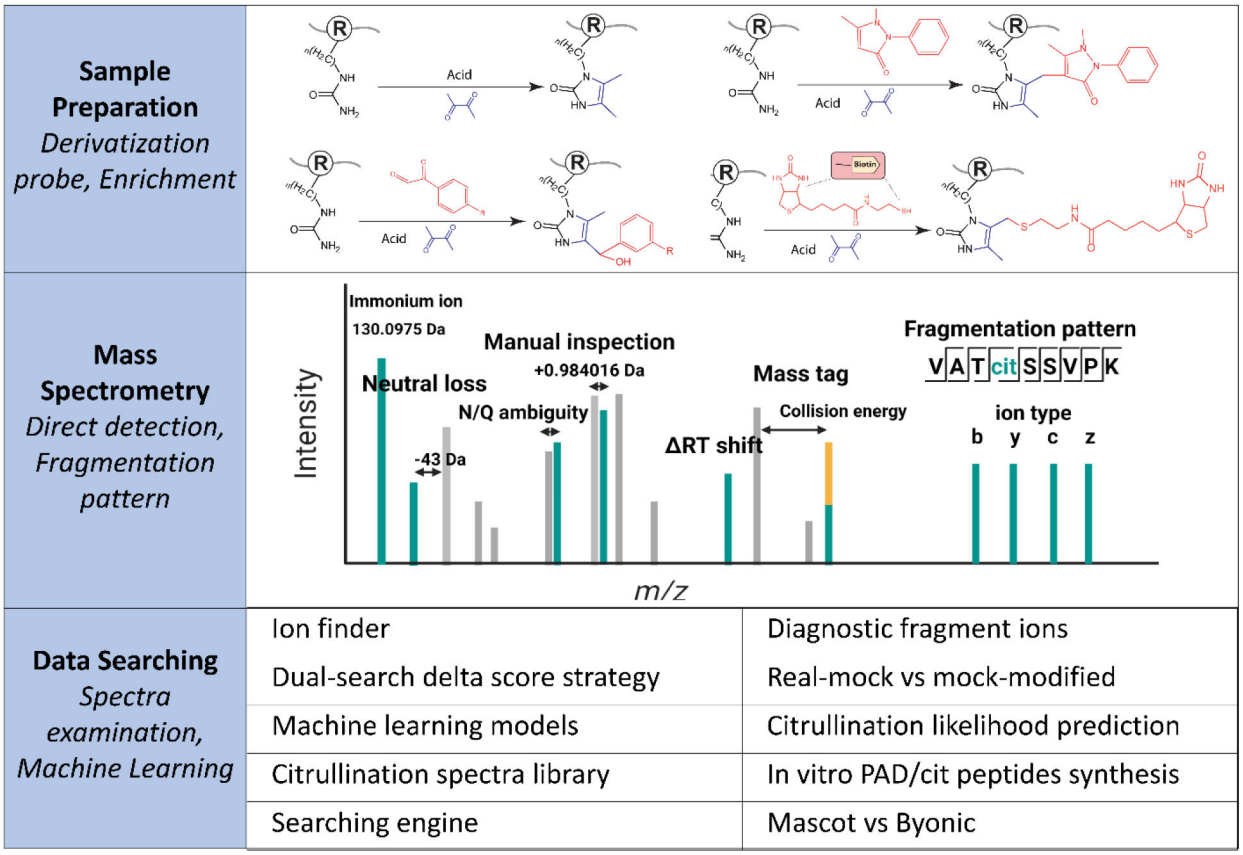

Figure 3.
Mass spectrometry approaches to analyze citrullinated proteins.

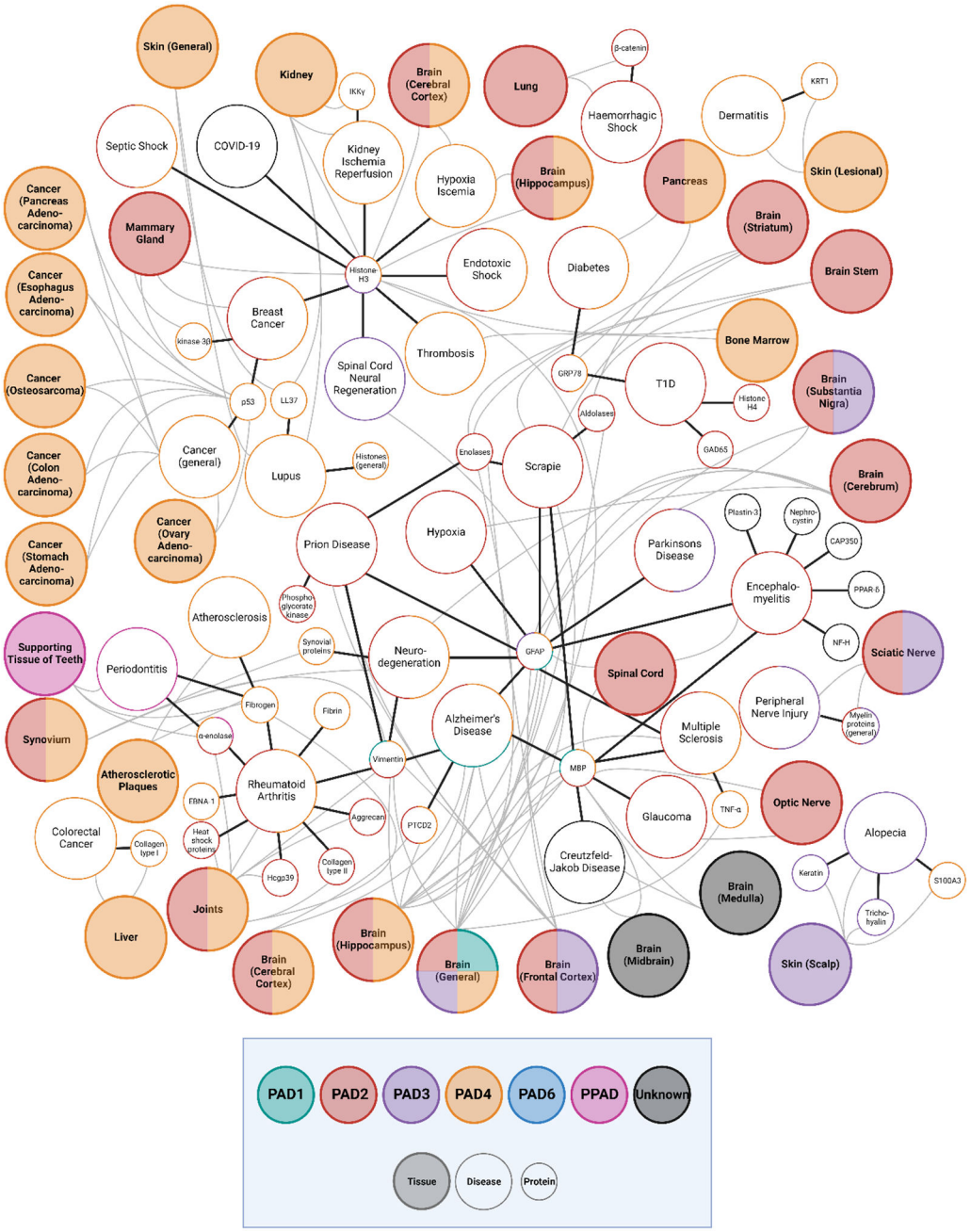

Figure 4.

Network of protein, tissues, and diseases implicated with citrullination, with citrullinated proteins reported in small circles, diseases reported in larger circles, and tissues reported in the large shaded circles. The PAD associated with each protein, tissue, and disease are indicated by the outline of the corresponding circle.